

PENTAPEPTIDE COMPOUNDS AND USES RELATED THERETO

TECHNICAL FIELD

The present invention is directed to biologically active organic compounds and precursors thereto, more specifically to pentapeptides and compositions containing these pentapeptides, and to methods for their use, *e.g.*, as cytotoxic agents.

BACKGROUND OF THE INVENTION

Several short peptidic compounds have been isolated from natural sources and found to have biological activity. Analogs of these compounds have also been prepared, and some were found to have biological activity. For example, Auristatin E (Pettit, G. R., Barkoczy, J. "Tumor inhibiting tetrapeptide bearing modified phenethyl amides" U.S. patent 5,635,483) is a synthetic analogue of the marine natural product, dolastatin 10, an agent that inhibits tubulin polymerization by binding to the same site on tubulin as the anticancer drug vincristine (Pettit, G. R. "The dolastatins" *Prog. Chem. Org.Nat. Prod.* 1997, 70, 1-79). Dolastatin 10, auristatin PE, and auristatin E are linear peptides comprised of four amino acids, three of which are unique to this class of compounds. Both dolastatin 10 and auristatin PE are in human clinical trials. The structural differences between the drugs reside in the C-terminal residue, in which the thiazolephenethyl amine of dolastatin 10 is replaced by a norephedrine unit in auristatin E.

The following references disclose dolastatin and auristatin compounds and analogs thereof.

"Preparation of peptide derivatives as dolastatin 10 analogs with antitumor effects" Sakakibara, Kyoichi; Gondo, Masaaki; Miyazaki, Koichi; Ito, Takeshi; Sugimura, Akihiro; Kobayashi, Motohiro. (Teikoku Hormone Mfg. Co., Ltd., Japan; Sakakibara, Kyoichi; Gondo, Masaaki; Miyazaki, Koichi; Ito, Takeshi; Sugimura, Akihiro; Kobayashi, Motohiro). PCT International Patent Publication No. WO 9633212 A1 (1996);

"Preparation of dolastatin 10 analogs as cancer inhibitory peptides" Pettit, George R.; Srirangam, Jayaram K. (Arizona Board of Regents, USA). PCT International Patent Publication No. WO 9614856 A1 (1996);

5 "Preparation of tetra- and pentapeptide dolastatin analogs as anticancer agents" Pettit, George R.; Srirangam, Jayaram K.; Williams, Michael D. (Arizona Board of Regents, USA). Eur. Pat. Appl. No. EP 695757 A2 (1996);

"Preparation of dolastatin 10 analog pentapeptide amides and esters as anticancer agents" Pettit, George R.; Srirangam, Jayaram K. (Arizona Board of Regents, USA). Eur. Pat. Appl. No. EP 695758 A2 (1996);

10 "Preparation of dolastatin analog pentapeptide methyl esters as anticancer agents" Pettit, George R.; Williams, Michael D.; Srirangam, Jayaram K. (Arizona Board of Regents, USA). Eur. Pat. Appl. No. EP 695759 A2 (1996);

"Preparation of novel peptide derivative as antitumor agent" Sakakibara, Kyoichi; Gondo, Masaaki; Miyazaki, Koichi; Ito, Takeshi; Sugimura, Akihiro; Kobayashi, Motohiro. (Teikoku Hormone Mfg. Co., Ltd., Japan). PCT International Patent Publication No. WO 9509864 A1 (1995);

"Preparation of tetrapeptide derivatives as antitumor agents" Sakakibara, Kyoichi; Gondo, Masaaki; Miyazaki, Koichi. (Teikoku Hormone Mfg. Co., Ltd., Japan). PCT International Patent Publication No. WO 9303054 A1 (1993);

20 "Antineoplastic agents 365. Dolastatin 10 SAR probes" Pettit, George R.; Srirangam, Jayaram K.; Barkoczy, Jozsef; Williams, Michael D.; Boyd, Michael R.; Hamel, Ernest; Pettit, Robin K.; Hogan, Fiona; Bai, Ruoli; Chapuis, Jean-Charles; McAllister, Shane C.; Schmidt, Jean M., *Anti-Cancer Drug Des.* (1998), 13(4), 243-277;

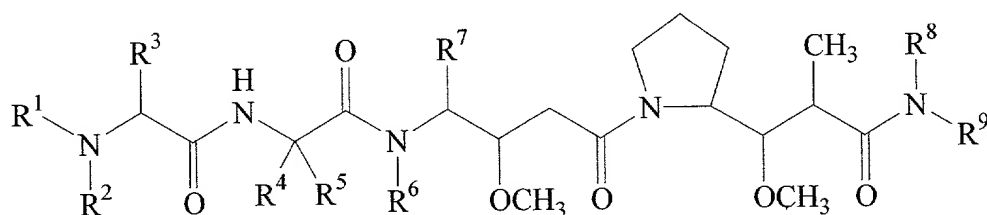
"Antineoplastic agents. 337. Synthesis of dolastatin 10 structural modifications" Pettit, George R.; Srirangam, Jayaram K.; Barkoczy, Jozsef; Williams, Michael D.; Durkin, Kieran P. M.; Boyd, Michael R.; Bai, Ruoli; Hamel, Ernest; Schmidt, Jean M.; Chapuis, Jean-Charles, *Anti-Cancer Drug Des.* (1995), 10(7), 529-44; and

"Synthesis and antitumor activity of novel dolastatin 10 analogs" Miyazaki, Koichi; Kobayashi, Motohiro; Natsume, Tsugitaka; Gondo, Masaaki; Mikami, Takashi; Sakakibara, Kyoichi; Tsukagoshi, Shigeru, *Chem. Pharm. Bull.* (1995), 43(10), 1706-18.

There is a need in the art for improved antitumor agents. Despite promising *in vitro* data for dolastatin 10 and its analogs, significant general toxicities at doses required for achieving a therapeutic affect compromise their efficacy in clinical studies. The present invention is directed to fulfilling this need and provides further advantages as disclosed herein.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a compound of the formula



wherein, independently at each location:

R¹ is selected from hydrogen and lower alkyl;

R² is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

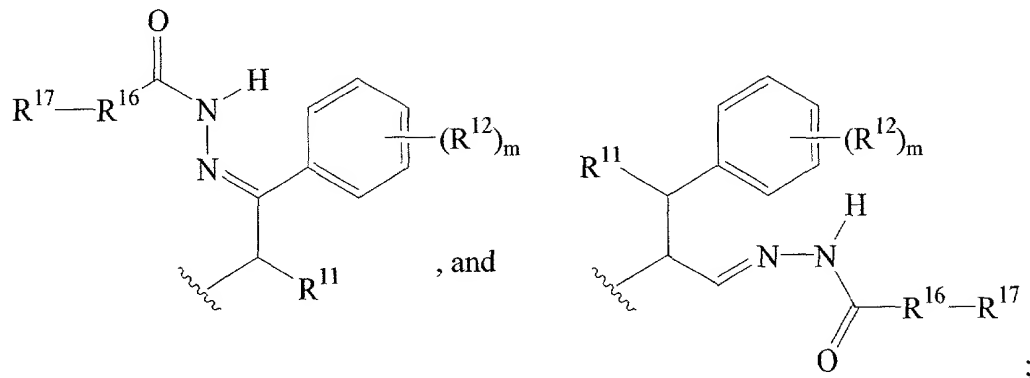
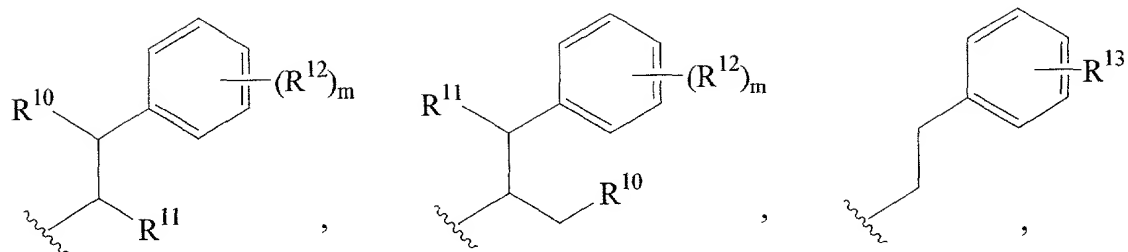
R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R⁶ is selected from hydrogen and lower alkyl;

R⁷ is *sec*-butyl or *iso*-butyl;

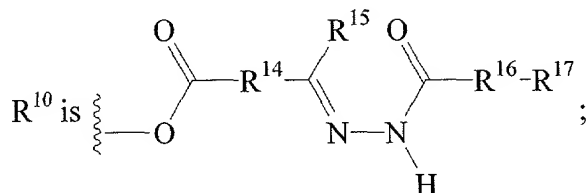
R⁸ is selected from hydrogen and lower alkyl; and

R⁹ is selected from



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wherein:

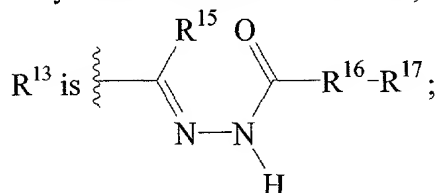


R^{11} is selected from hydrogen and lower alkyl;

R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5

where R^{12} is independently selected at each occurrence; and

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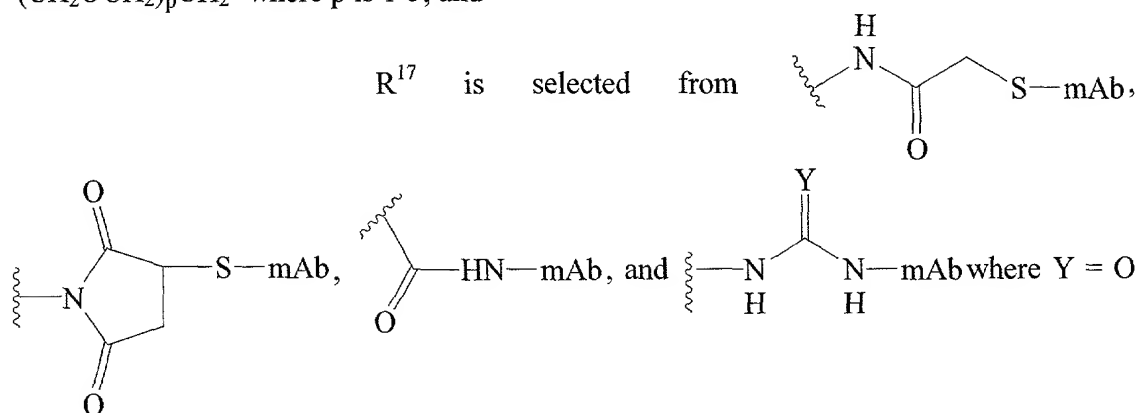


wherein:

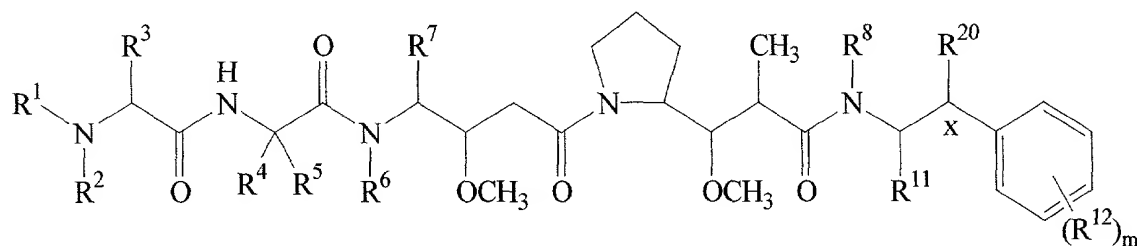
R^{14} is selected from a direct bond, divalent lower alkyl and divalent aryl;

R^{15} is selected from hydrogen, lower alkyl and aryl;

R^{16} is selected from divalent lower alkyl, divalent aryl, and $-(CH_2OCH_2)_pCH_2-$ where p is 1-5; and



In another aspect, the present invention provides a compound of the formula



wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-CH_2-C_{5-7}$ carbocycle when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula

$-(CR^aR^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R^6 is selected from hydrogen and lower alkyl;

R^7 is *sec*-butyl or *iso*-butyl;

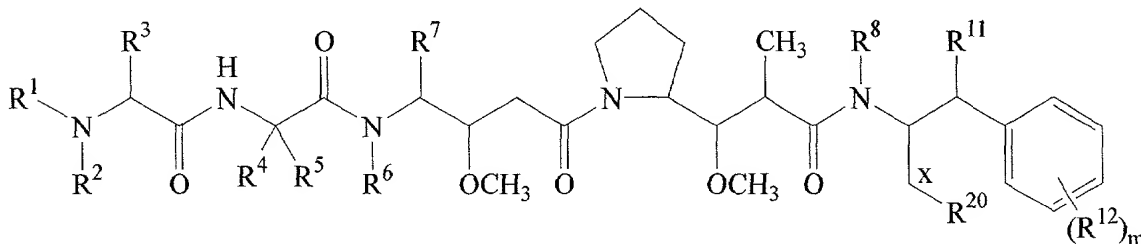
R^8 is selected from hydrogen and lower alkyl;

R^{11} is selected from hydrogen and lower alkyl;

R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where R^{12} is independently selected at each occurrence; and

R^{20} is a reactive linker group having a reactive site that allows R^{20} to be reacted with a targeting moiety, where R^{20} can be bonded to the carbon labeled "x" by either a single or double bond.

In another aspect, the present invention provides a compound of the formula



wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R^6 is selected from hydrogen and lower alkyl;

R^7 is *sec*-butyl or *iso*-butyl;

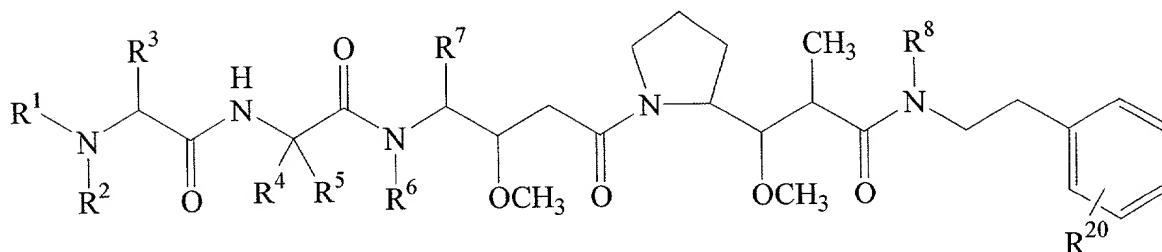
R^8 is selected from hydrogen and lower alkyl;

R^{11} is selected from hydrogen and lower alkyl;

R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where R^{12} is independently selected at each occurrence; and

R^{20} is a reactive linker group having a reactive site that allows R^{20} to be reacted with a targeting moiety, where R^{20} can be bonded to the carbon labeled "x" by either a single or double bond.

In another aspect, the present invention provides a compound of the formula



wherein, independently at each location:

5 R¹ is selected from hydrogen and lower alkyl;

R² is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is
 10 selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula
 -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl
 and n is selected from 2, 3, 4, 5 and 6;

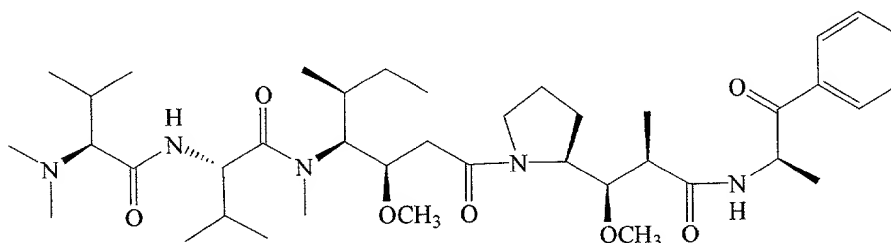
R⁶ is selected from hydrogen and lower alkyl;

R⁷ is *sec*-butyl or *iso*-butyl;

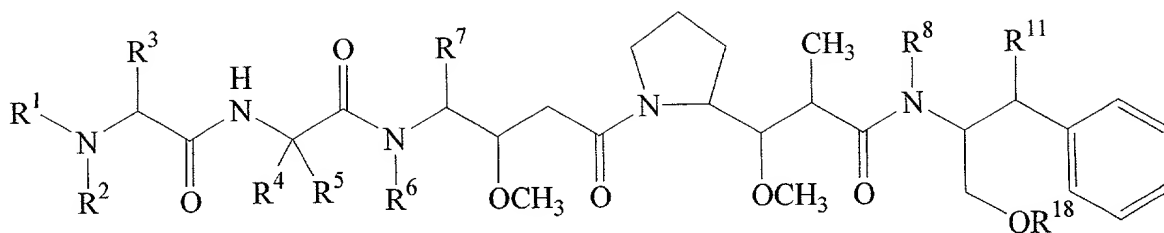
R⁸ is selected from hydrogen and lower alkyl; and

15 R²⁰ is a reactive linker group comprising a reactive site that allows R²⁰ to be
 reacted with a targeting moiety.

In another aspect, the present invention provides a compound of the formula



In another aspect, the present invention provides a compound of the formula



wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

5 R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-CH_2-C_{5-7}$ carbocycle when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(CR^aR^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl
10 and n is selected from 2, 3, 4, 5 and 6;

R^6 is selected from hydrogen and lower alkyl;

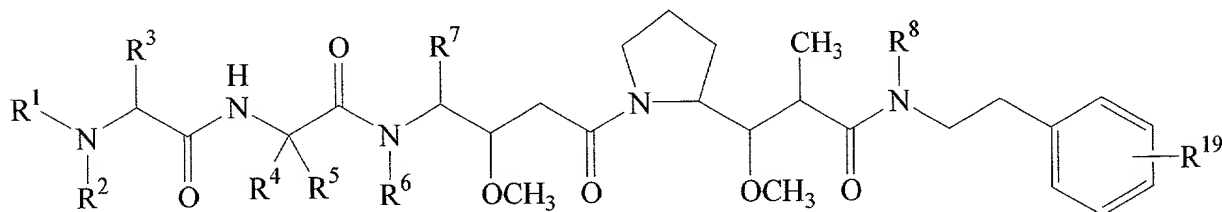
R^7 is *sec*-butyl or *iso*-butyl;

R^8 is selected from hydrogen and lower alkyl;

R^{11} is selected from hydrogen and lower alkyl; and

15 R^{18} is selected from hydrogen, a hydroxyl protecting group, and a direct bond where OR^{18} represents =O.

In another aspect, the present invention provides a compound of the formula



20 wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

5 R⁶ is selected from hydrogen and lower alkyl;

R⁷ is *sec*-butyl or *iso*-butyl;

R⁸ is selected from hydrogen and lower alkyl; and

R¹⁹ is selected from hydroxy- and oxo-substituted lower alkyl.

10 In another aspect, the present invention provides a composition comprising a compound as described above and a pharmaceutically acceptable carrier, diluent or excipient.

In another aspect, the present invention provides a method for killing a cell, the method comprising administering to the cell a lethal amount of a compound or composition as described above.

15 In another aspect, the present invention provides a method of killing a cell comprising

a. delivering a compound or composition as described above to a cell, where the compound enters the cell;

b. cleaving mAb from the remainder of the compound; and

20 c. killing the cell with the remainder of the compound.

In another aspect, the present invention provides a method of killing or inhibiting the multiplication of tumor cells or cancer cells in a human or other animal, the method comprising administering to the human or animal a therapeutically effective amount of a compound or composition described above.

25 These and related aspects of the present invention are described in more detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a synthetic scheme illustrating the preparation of specific tripeptides useful in preparing compounds of the present invention.

Figure 2 shows a synthetic scheme illustrating the preparation of specific dipeptides useful in preparing compound of the present invention.

Figures 3, 4 and 5 show synthetic schemes illustrating the preparation of pentapeptides from tripeptides and dipeptides

Figures 6, 7 and 8 show synthetic schemes for conjugating a pentapeptide to a heterobifunctional linker to provide a drug-reactive linker conjugate.

Figures 9, 10 and 11 show synthetic schemes for conjugating a ligand, and specifically a mAb, to a drug-reactive linker conjugate to provide prodrugs of the present invention.

Figures 12A, 12B and 12C show the *in vitro* cytotoxicity of drugs and mAb-drug conjugates on (A) and (B) L2987 human lung adenoma cells, and (A) and (C) Kato III human gastric carcinoma cells.

Figures 13A and 13B show the cytotoxic effects of auristatin E and auristatin E-containing conjugates on L2987 human lung adenocarcinoma cells (Figure 13A) and various hematologic cell lines treated with AC10-S-40a (Figure 13B).

Figures 14A and 14B show median tumor volume observed when nude mice with subcutaneous L2987 human lung adenocarcinoma or 3396 human breast carcinoma xenografts were injected with conjugates or drug according to the schedule shown in the two Figures.

DETAILED DESCRIPTION OF THE INVENTION

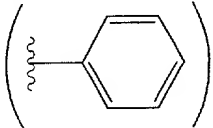
Among other aspects, the present invention provides short peptide-containing compounds having biological activity, *e.g.*, cytotoxicity, and methods for their use, *e.g.*, in cancer therapy. Before describing the compounds of the present invention, reference is made to the following definitions that are used herein.

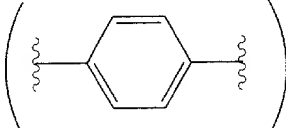
Definitions

As used herein the following terms have the indicated meanings:

"Animal subjects" include humans, monkeys, pigs, goats, cows, horses, dogs, and cats. Birds, including fowl, are also a suitable animal subject.

- 5 "Aryl" refers to aromatic groups which have at least one ring having a conjugated pi electron system and includes carbocyclic aryl and heterocyclic aryl. "Carbocyclic aryl" refers to aromatic groups wherein the ring atoms on the aromatic ring are carbon atoms. Carbocyclic aryl groups include monocyclic carbocyclic aryl groups and polycyclic groups, all of which may be optionally substituted. Suitable carbocyclic aryl
- 10 groups include phenyl and naphthyl. Suitable substituted carbocyclic aryl groups include indene and phenyl substituted by one to two substituents such as lower alkyl, hydroxy, lower alkoxy, lower alkoxycarbonyl, halogen, trifluoromethyl, nitro, and cyano. Substituted naphthyl refers to 1- or 2-naphthyl substituted by lower alkyl, lower alkoxy, or halogen. "Heterocyclic aryl", which may also be called "heteroaryl" refers to aryl groups
- 15 having from 1 to 9 carbon atoms and the remainder of the atoms are heteroatoms, and includes those heterocyclic systems described in "Handbook of Chemistry and Physics," 49th edition, 1968, R.C. Weast, editor; The Chemical Rubber Co., Cleveland, OH. See particularly Section C, Rules for Naming Organic Compounds, B. Fundamental Heterocyclic Systems. Suitable heteroatoms include oxygen, nitrogen, and S(O)_i, wherein i
- 20 is 0, 1 or 2. Suitable examples of heteroaryl groups include, without limitation, benzofuran, benzothiophene, indole, benzopyrazole, coumarin, isoquinoline, pyrrole, thiophene, furan, thiazole, imidazole, pyrazole, triazole, quinoline, pyrimidine, pyridine, pyridone, pyrazine, pyridazine, isothiazole, isoxazole and tetrazole. "Monovalent aryl groups" are bonded to one other group, while "divalent aryl" refers to an aryl group that is

- 25 bonded to two other groups. For example, phenyl  is a monovalent aryl

group, while phenylene  is one example of a divalent aryl group.

"Carbocycle" or "carbocyclic ring" refers to any saturated, unsaturated or aromatic monocyclic ring where the ring atoms are carbon. A C₅₋₇carbocycle refers to any carbocyclic ring having 5, 6 or 7 carbon atoms that form the ring. Examples of such carbocycles include, but are not limited to, cyclopentyl, cyclohexyl, cyclohexenyl, cyclohexadienyl, cycloheptyl, and phenyl.

"Direct bond" refers to a pair of electrons that hold together two adjacent atoms. Thus, when two atoms are separated by a direct bond, those two atoms are bonded together by a covalent bond.

"Leaving group" refers to a functional group that can be readily substituted by another functional group. A preferred leaving group is replaced by S_N2 displacement by a nucleophile. Such leaving groups are well known in the art, and include halides (e.g., chloride, bromide, iodide), mesylate, tosylate, etc.

"Lower alkyl" refers to a straight or a branched chain, cyclic or acyclic, monovalent saturated hydrocarbon, halocarbon or hydrohalocarbon radical of one to ten carbon atoms. Thus, the alkyl group may optionally have halogen substitution, where a halocarbon contains only carbon and halogen, where a hydrohalocarbon contains carbon, halogen and hydrogen. Exemplary lower alkyl groups are, without limitation, methyl, ethyl, propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, tert-butyl, n-pentyl, n-hexyl, and cyclohexyl. "Divalent lower alkyl" refers to a lower alkyl group that is bonded to two groups. For example, methyl (-CH₃) is a monovalent lower alkyl group, while methylene (-CH₂-) is a divalent lower alkyl group. A hydroxy- or oxo-substituted lower alkyl refers to a lower alkyl group wherein a hydrogen on the lower alkyl group is replaced by -OH (for a hydroxy-substituted lower alkyl), or two hydrogens on a single carbon of the lower alkyl group are replaced by =O (for an oxo-substituted lower alkyl).

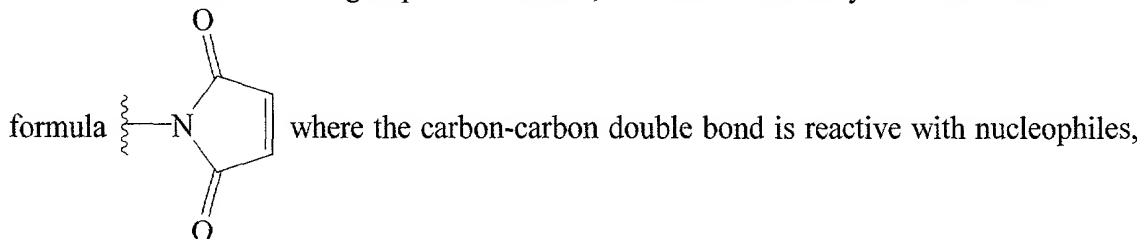
"mAb" refers to monoclonal antibody. Monoclonal antibodies are homogeneous with respect to immunoglobulin heavy and light chains. Such antibodies are well known in the art. See, e.g., Harlow et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988). Monoclonal antibodies include chimeric antibodies, where a chimeric antibody has at least one constant region domain derived from a first

mammalian species and at least one variable region domain derived from a second, distinct mammalian species. See, e.g., Morrison et al., 1984, Proc. Natl. Acad. Sci. USA, 81:6851-55. The chimeric antibody may be, for example, a humanized chimeric antibody. See, e.g., Jones et al., 1986 Nature 321:522-25; Riechmann et al., 1988 Nature 332:323-27; and Bajorath et al., 1995 Ther. Immunol. 2:95-103; EP-0578515-A3. The monoclonal antibody may be constructed as single chain Fv polypeptide fragments (single chain antibodies). See, e.g., Bird et al., 1988 Science 242:423-426; Huston et al., 1988 Proc. Natl. Acad. Sci. USA 85:5879-5883.

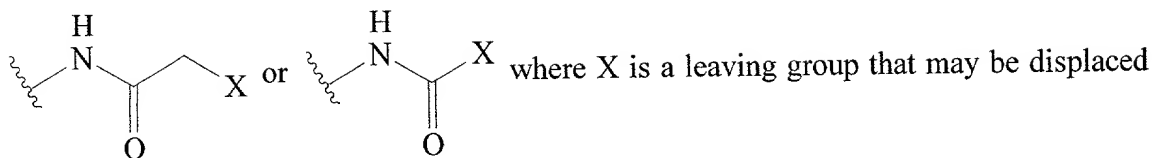
The following abbreviations may be used herein and have the indicated definitions: *t*-Boc is *tert*-butoxy carbonyl, DCC is dicyclohexylcarbodiimide, Fmoc is 9-fluorenylmethoxycarbonyl, DEPC is diethyl phosphorocyanidate, DMAP is 4-(*N,N*-dimethylamino) pyridine, PyBrop is bromo-tris-pyrrolidino-phosphonium hexafluorophosphate, TEAA is triethylammonium acetate, TFA is trifluoroacetic acid, and Z is carbobenzyloxy.

In the phrase “R²⁰ is a reactive linker having a reactive site that allows R²⁰ to be reacted with a targeting moiety”, a “reactive site” refers to a functional moiety that can undergo reaction with a ligand so as to provide a covalent bond between the ligand and the reactive linker. In one aspect of the invention, the reactive site is reactive with a functional group selected from thiol and amino. Both thiol and amino groups are present in proteins, where thiol groups are, e.g., produced by reduction of disulfide bonds in proteins, and amino groups are present in, e.g., a lysine moiety of a protein.

In a preferred aspect of the invention, the reactive linker has a reactive site that is reactive with a thiol group. For instance, the reactive site may be a maleimide of the



e.g., thiol groups. Alternatively, the reactive site may have the formulae

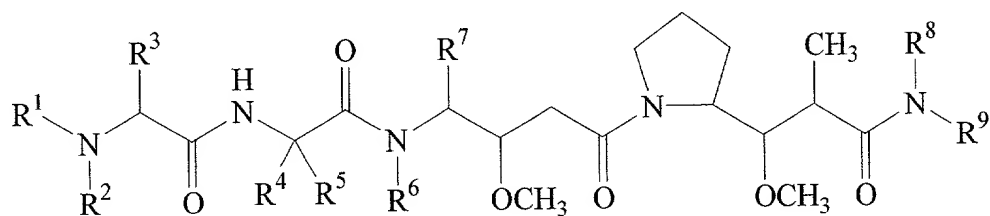


by a nucleophile, *e.g.*, a thiol group as found on a protein.

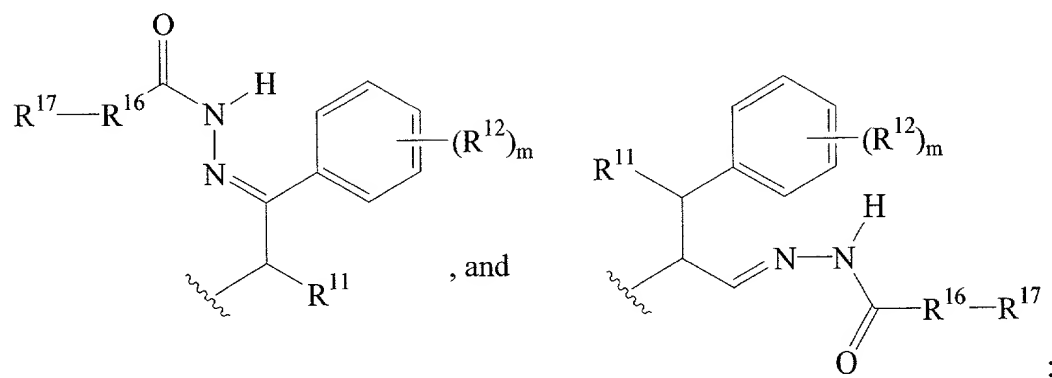
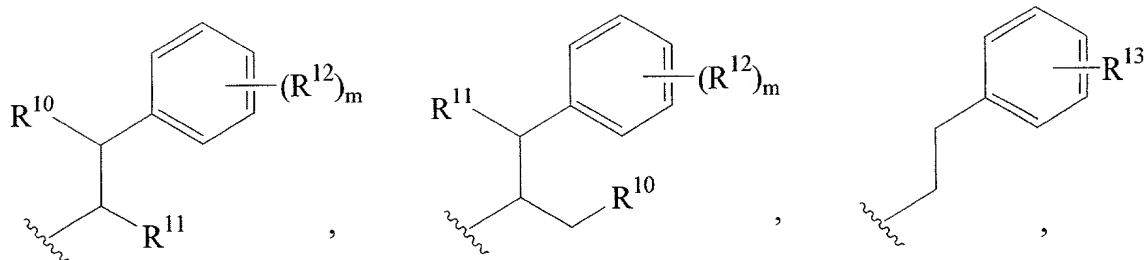
In another preferred aspect of the invention, the reactive linker contains a reactive site that is reactive with an amine or amino group. Suitable amine reactive sites include, without limitation, activated esters such as *N*-hydroxysuccinimide esters, *p*-nitrophenyl esters, pentafluorophenyl esters, and other reactive functionalities, such as isothiocyanates, isocyanates, anhydrides, acid chlorides, and sulfonyl chlorides. Each of these functionalities provides a reactive site for a reactive linker of the present invention.

Compounds

In one aspect, the present invention provides compounds of the general structure "drug-linker-targeting agent", where the drug is a pentapeptide as disclosed herein and the targeting agent is a monoclonal antibody (mAb). Such compounds have the following structure, and may also be referred to herein as prodrugs.



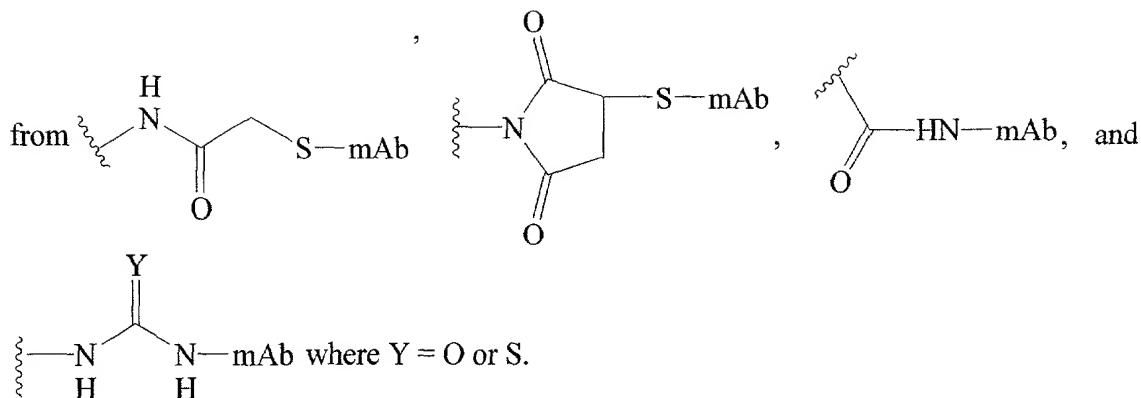
In this structure, independently at each location: R¹ is selected from hydrogen and lower alkyl; R² is selected from hydrogen and lower alkyl; R³ is lower alkyl; R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R⁶ is selected from hydrogen and lower alkyl; R⁷ is *sec*-butyl or *iso*-butyl; R⁸ is selected from hydrogen and lower alkyl; and R⁹ is selected from



wherein R^{10} is: R^{14} is selected from hydrogen and

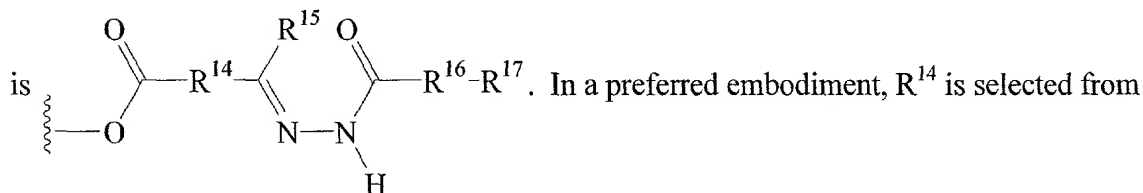
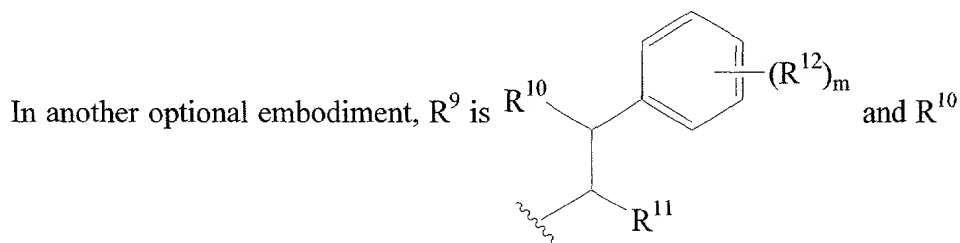
5 lower alkyl; R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where

R^{12} is independently selected at each occurrence; and R^{13} is R^{15} is selected from hydrogen, lower alkyl and aryl; R^{16} is selected from divalent lower alkyl, divalent aryl, and $-(CH_2OCH_2)_pCH_2-$ where p is 1-5; and R^{17} is selected



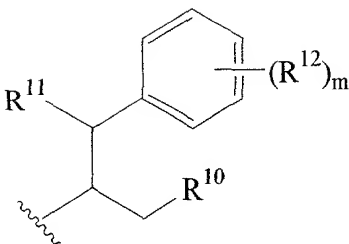
In optional embodiments of the invention: R¹ is hydrogen; R¹ and R² are methyl; R³ is isopropyl; R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle and R⁵ is selected from H and methyl; R⁴ is selected from lower alkyl, and R⁵ is selected from H and methyl; R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R⁶ is lower alkyl; R⁸ is hydrogen.

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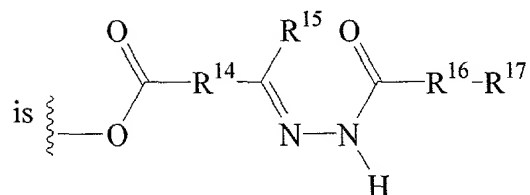


divalent aryl and divalent alkyl; R¹⁵ is selected from lower alkyl and aryl; and R¹⁶ is divalent lower alkyl.

In another optional embodiment, R⁹ is



and R¹⁰

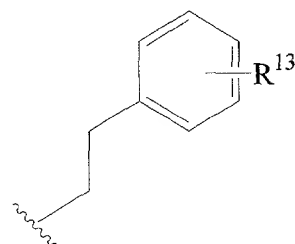


. In a preferred embodiment, R¹⁴ is selected from

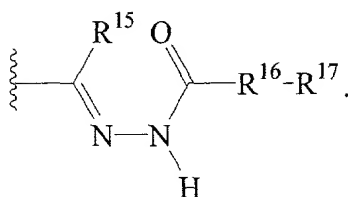
divalent aryl and divalent alkyl; R¹⁵ is selected from lower alkyl and aryl; and R¹⁶ is divalent lower alkyl.

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In another optional embodiment, R⁹ is



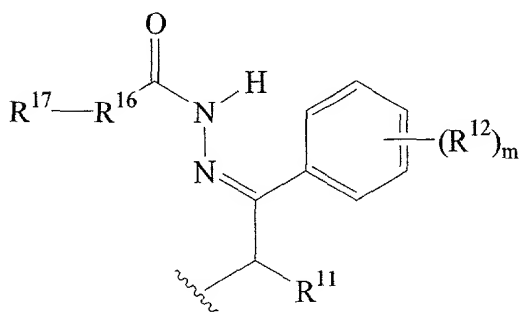
and R¹³ is



. In a preferred embodiment, R¹⁵ is lower alkyl; and R¹⁶ is

divalent lower alkyl.

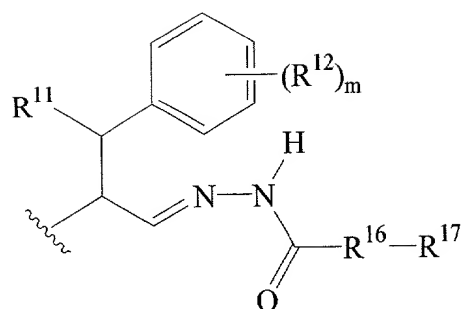
In another optional embodiment, R⁹ is



. In a preferred embodiment, R¹⁶ is divalent

10 lower alkyl.

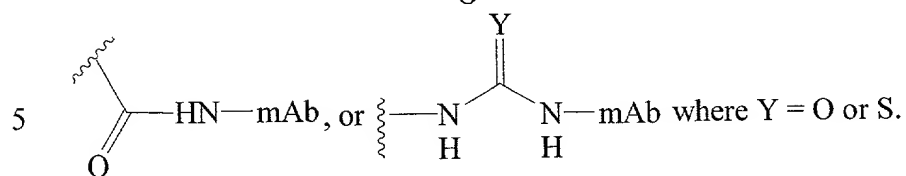
In another optional embodiment, R⁹ is



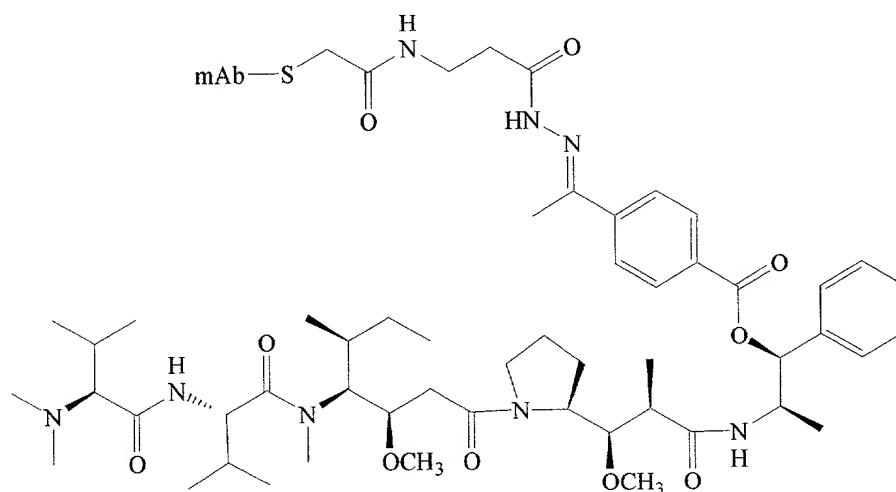
In a preferred embodiment, R¹⁶ is selected from divalent lower alkyl and divalent aryl.

In one embodiment R¹⁷ is In another

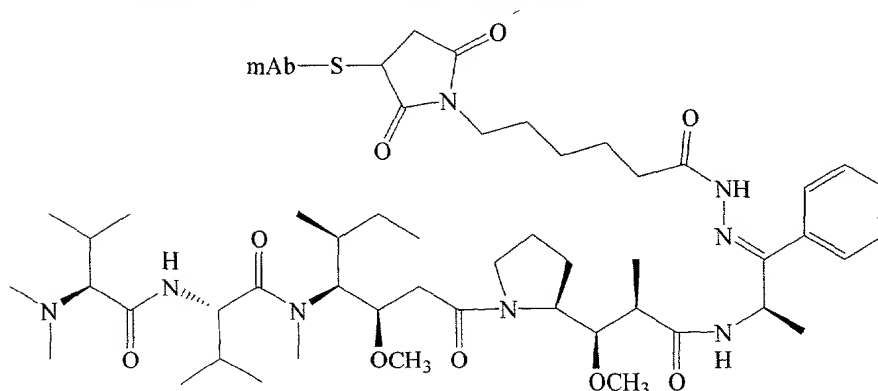
embodiment, R¹⁷ is . In another embodiment, R¹⁷ is



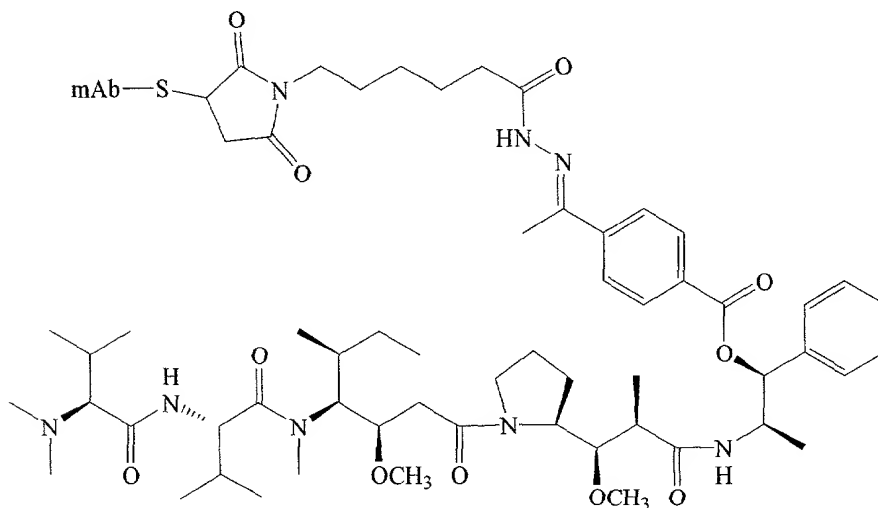
For example, the present invention provides a prodrug of the formula



In addition, the present invention provides a prodrug of the formula:

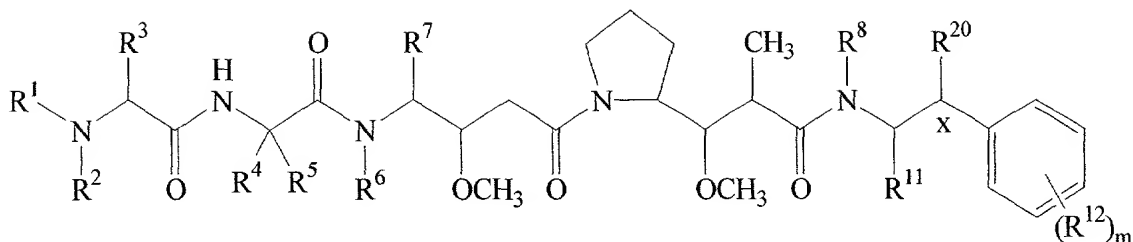


In addition, the present invention provides a prodrug of the formula:



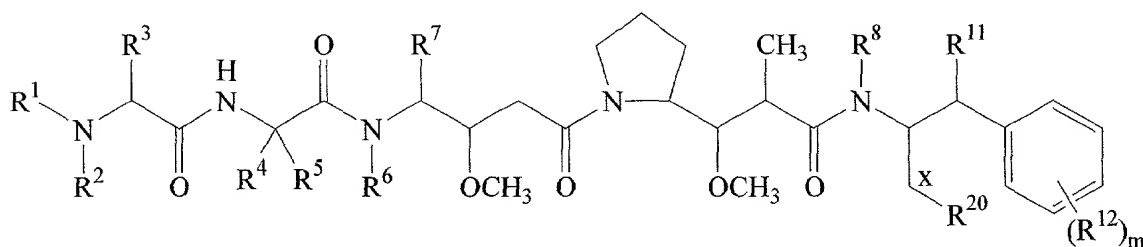
5 In addition to prodrugs as described above, the present invention also provides intermediate compound that can be used to prepare prodrugs. These intermediate compounds contain a reactive linker group R^{20} having a reactive site that allows R^{20} to be reacted with a targeting moiety.

In one aspect, the intermediate compounds has the formula



wherein, independently at each location: R¹ is selected from hydrogen and lower alkyl; R² is selected from hydrogen and lower alkyl; R³ is lower alkyl; R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R⁶ is selected from hydrogen and lower alkyl; R⁷ is *sec*-butyl or *iso*-butyl; R⁸ is selected from hydrogen and lower alkyl; R¹¹ is selected from hydrogen and lower alkyl; R¹² is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where R¹² is independently selected at each occurrence; and R²⁰ is a reactive linker group having a reactive site that allows R²⁰ to be reacted with a targeting moiety, where R²⁰ can be bonded to the carbon labeled "x" by either a single or double bond.

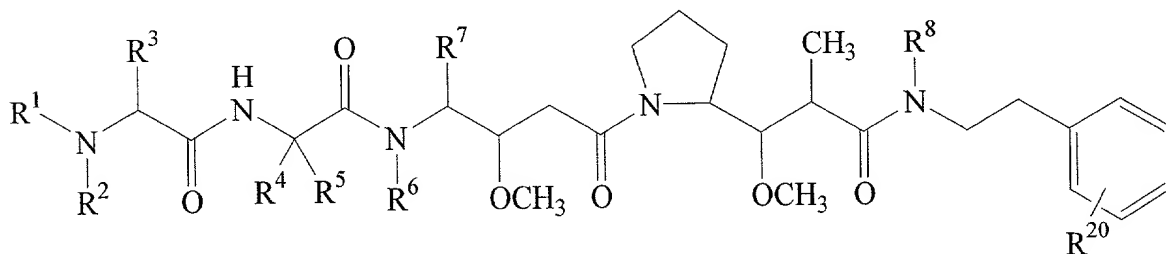
In another aspect, the intermediate compounds has the formula



wherein, independently at each location: R¹ is selected from hydrogen and lower alkyl; R² is selected from hydrogen and lower alkyl; R³ is lower alkyl; R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R⁶ is selected from hydrogen and lower alkyl; R⁷ is *sec*-butyl or *iso*-butyl; R⁸ is selected

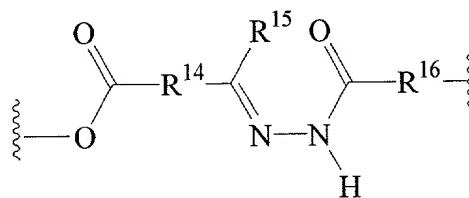
from hydrogen and lower alkyl; R^{11} is selected from hydrogen and lower alkyl; R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where R^{12} is independently selected at each occurrence; and R^{20} is a reactive linker group having a reactive site that allows R^{20} to be reacted with a targeting moiety, where R^{20} can be bonded to the carbon labeled "x" by either a single or double bond.

In another aspect, the intermediate compounds has the formula



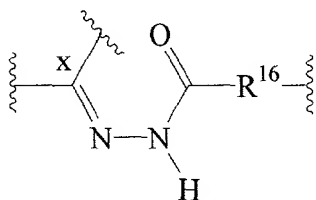
wherein, independently at each location: R^1 is selected from hydrogen and lower alkyl; R^2 is selected from hydrogen and lower alkyl; R^3 is lower alkyl; R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2\text{-C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R^6 is selected from hydrogen and lower alkyl; R^7 is *sec*-butyl or *iso*-butyl; R^8 is selected from hydrogen and lower alkyl; and R^{20} is a reactive linker group comprising a reactive site that allows R^{20} to be reacted with a targeting moiety.

In another aspect, R^{20} comprises a hydrazone of the formula



wherein: R^{14} is selected from a direct bond, divalent lower alkyl and divalent aryl; R^{15} is selected from hydrogen, lower alkyl, and aryl; and R^{16} is selected from divalent lower alkyl, divalent aryl, and $-(\text{CH}_2\text{OCH}_2)_p\text{CH}_2-$ where p is 1-5.

In another aspect, R²⁰ comprises a hydrazone of the formula:



wherein R¹⁶ is selected from divalent lower alkyl, divalent aryl, and $-(CH_2OCH_2)_pCH_2-$ where p is 1-5, and x identifies the carbon also marked x in the intermediate structures
5 shown above.

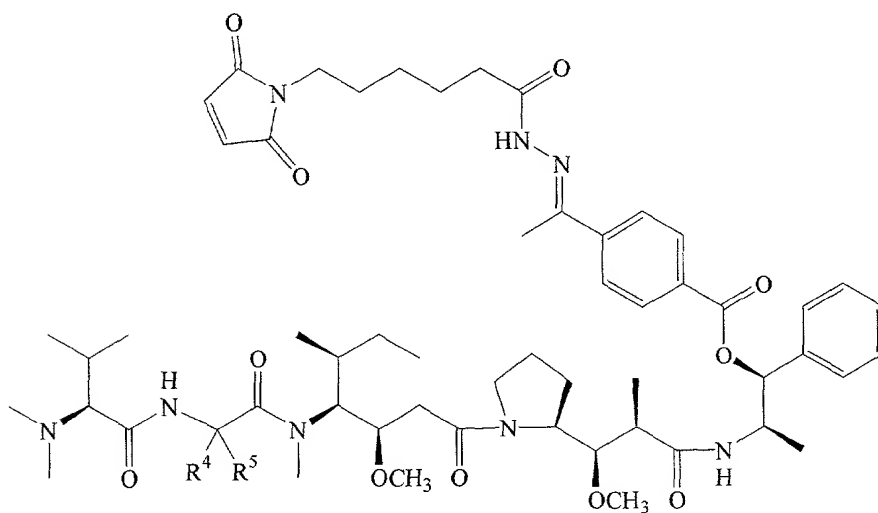
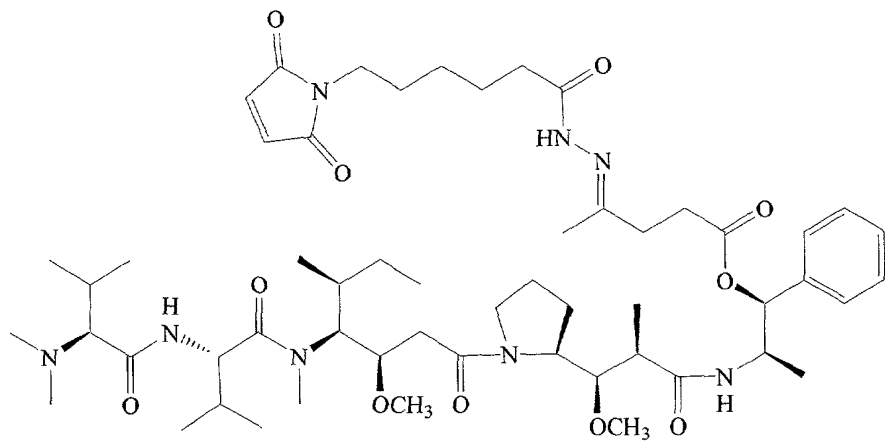
In one aspect, optionally in addition to one of the foregoing hydrazone

structures, R²⁰ comprises a reactive site having the formula . In another

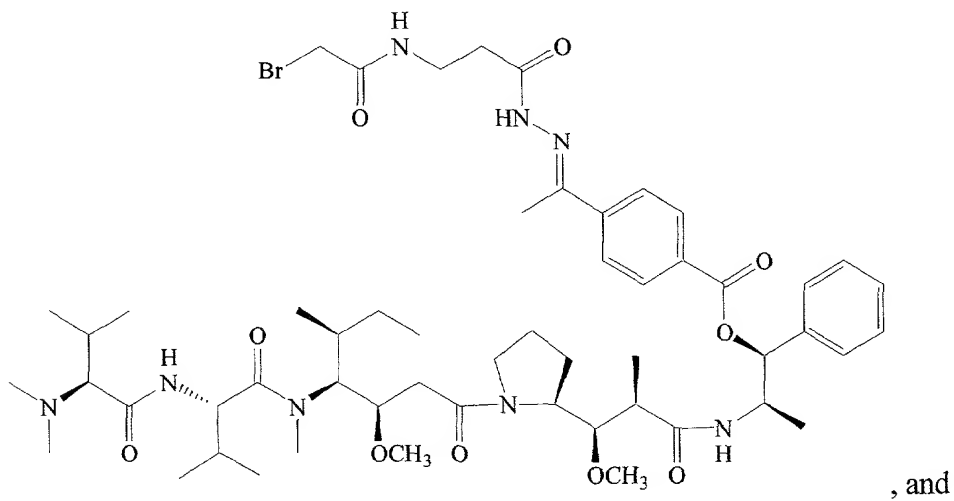
aspect, again optionally in addition to one of the foregoing hydrazone structures, R²⁰ comprises a reactive site having the formula wherein X is a leaving

10 group. In another aspect, again optionally in addition to one of the foregoing hydrazone structures, R²⁰ comprises a reactive site having the formula wherein X is a leaving group.

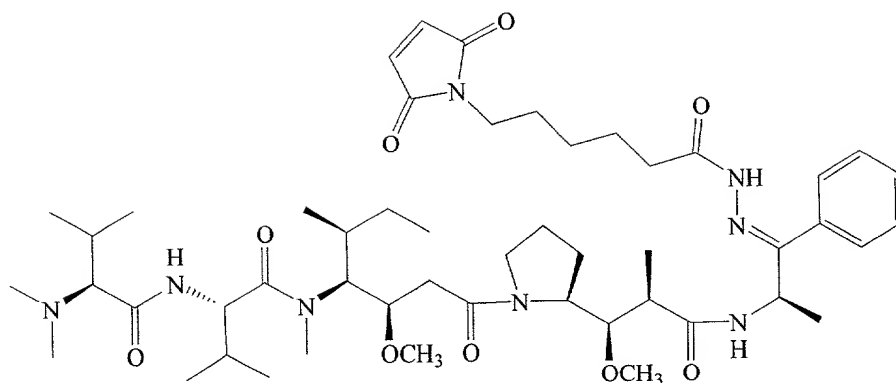
For example, the present invention provides intermediate compounds having the structures:



wherein R^4 is selected from *iso*-propyl and *sec*-butyl, and R^5 is hydrogen;

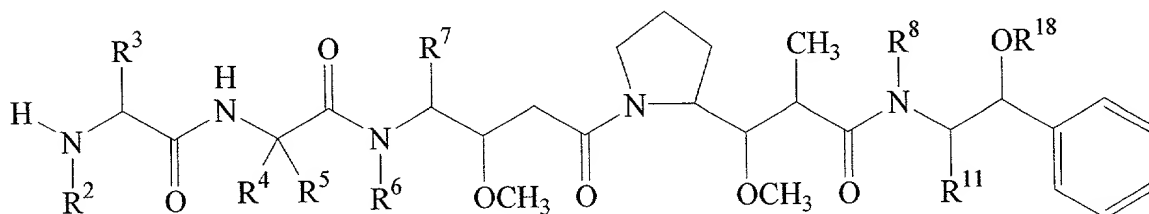


, and



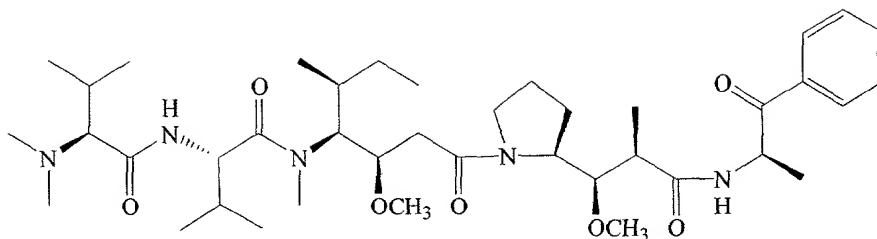
In addition to prodrugs as described above, and precursor intermediate compounds to the prodrugs, the present invention provides pentapeptide drugs of the following structures.

5 Thus, in one embodiment, the present invention provides a compound of the formula

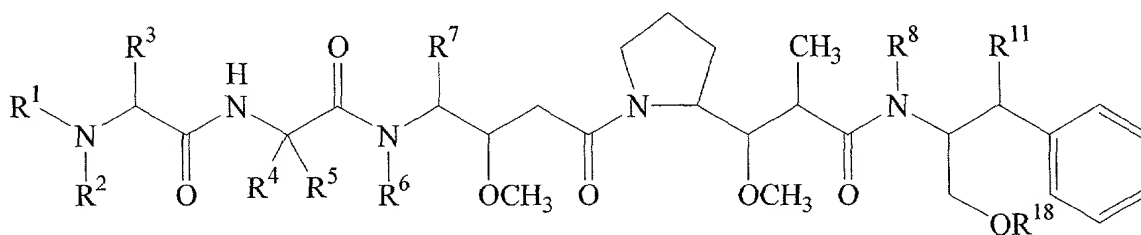


wherein, independently at each location: R^2 is selected from hydrogen and lower alkyl; R^3 is lower alkyl; R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R^6 is selected from hydrogen and lower alkyl; R^7 is *sec*-butyl or *iso*-butyl; R^8 is selected from hydrogen and lower alkyl; R^{11} is selected from hydrogen and lower alkyl; and R^{18} is selected from hydrogen, a hydroxyl protecting group, and a direct bond where OR^{18} represents $=\text{O}$.

For instance, the present invention provides compound of the formula

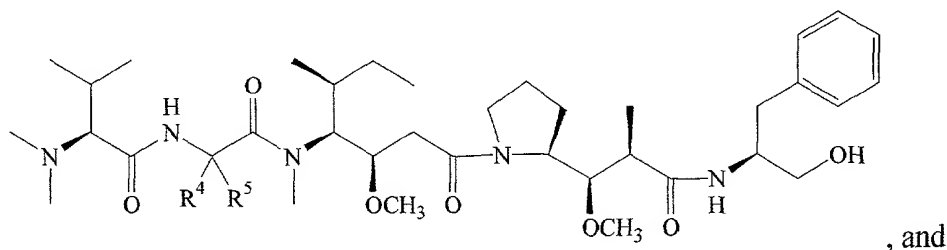


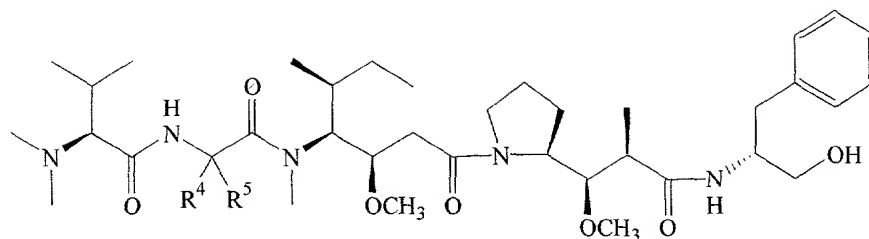
In another embodiment, the present invention provides a compound of the formula



wherein, independently at each location: R^1 is selected from hydrogen and lower alkyl; R^2 is selected from hydrogen and lower alkyl; R^3 is lower alkyl; R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2\text{-C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R^6 is selected from hydrogen and lower alkyl; R^7 is *sec*-butyl or *iso*-butyl; R^8 is selected from hydrogen and lower alkyl; R^{11} is selected from hydrogen and lower alkyl; and R^{18} is selected from hydrogen, a hydroxyl protecting group, and a direct bond where OR^{18} represents $=\text{O}$.

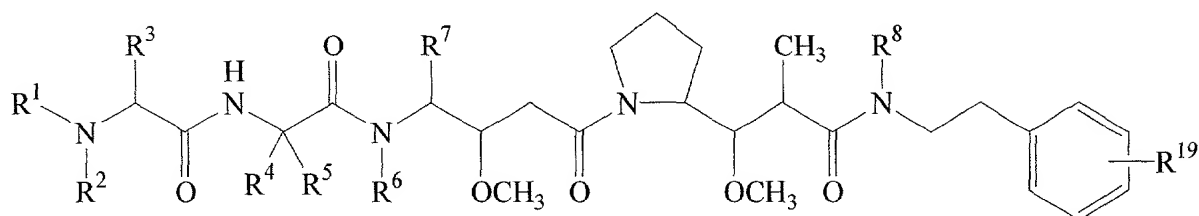
For instance, the present invention provides a compounds of the formulae





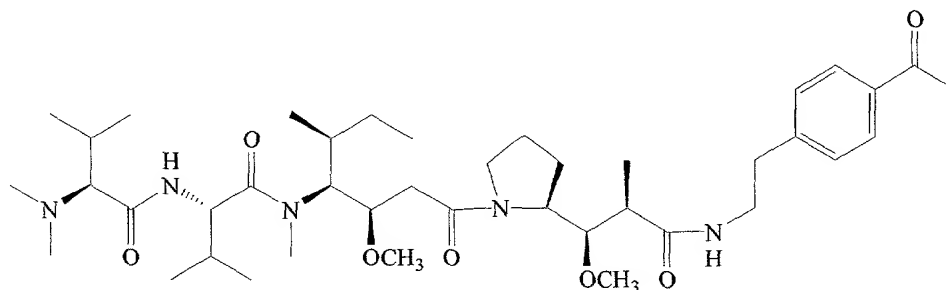
wherein R⁴ *iso*-propyl and R⁵ is hydrogen.

In another embodiment, the present invention provides a compound of the formula



wherein, independently at each location: R¹ is selected from hydrogen and lower alkyl; R² is selected from hydrogen and lower alkyl; R³ is lower alkyl; R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; R⁶ is selected from hydrogen and lower alkyl; R⁷ is *sec*-butyl or *iso*-butyl; R⁸ is selected from hydrogen and lower alkyl; and R¹⁹ is selected from hydroxy- and oxo-substituted lower alkyl.

For instance, the present invention provides a compound of the formula

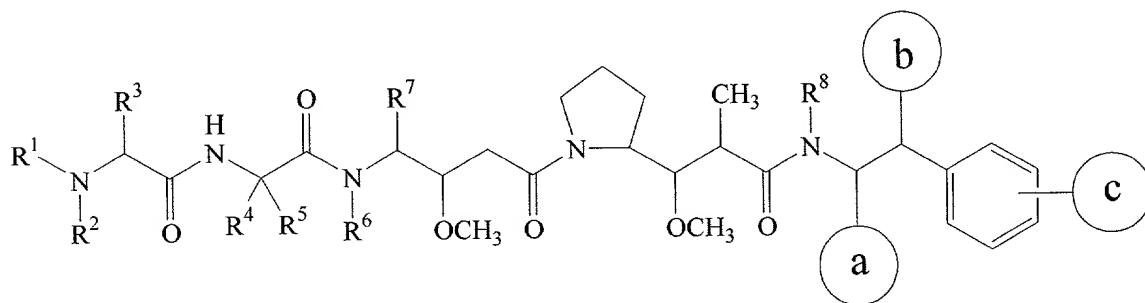


In various embodiment, the pentapeptide drugs of the invention have a pentapeptide structure as shown above, wherein: R^1 is hydrogen; or R^1 and R^2 are methyl; and/or R^3 is isopropyl; and/or R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_5-$ carbocycle and R^5 is selected from H and methyl; or R^4 is selected from lower alkyl, and
 5 R^5 is selected from H and methyl; or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6; and/or R^6 is lower alkyl; and/or R^8 is hydrogen; and/or R^{11} is hydrogen; and/or $-\text{OR}^{18}$ is $=\text{O}$ or R^{19} is oxo-substituted lower alkyl.

10 General Synthesis

In one aspect, the present invention provides a drug-linker-ligand conjugate as well as precursors thereto. The drug-linker-conjugate is designed so that upon delivery of the conjugate to a cell, and particularly a tumor cell, the conjugate will partially degrade in a manner that frees the drug, and perhaps some residue from the linker that remains
 15 attached to the drug, from the ligand and typically some residue of the linker that remains attached to the ligand. The drug (or drug-linker residue) will then act in a cytotoxic manner to kill the tumor cells. The drug-linker-conjugate may be referred to herein as a prodrug. The ligand is typically a targeting moiety, that will localize the prodrug in the vicinity of a tumor cell.

20 The drug has the basic structure shown below:



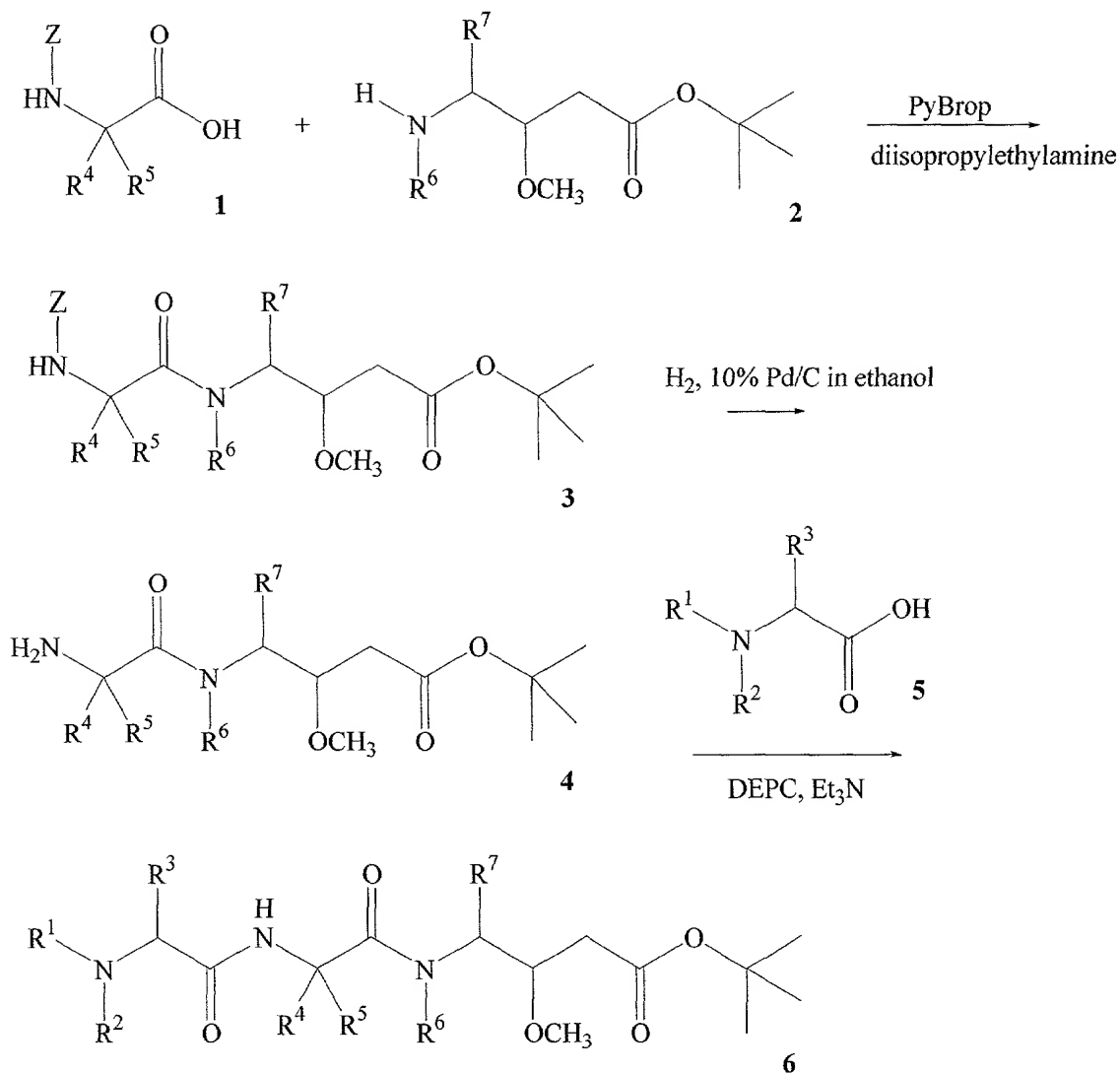
where the $-\text{linker-ligand}$ is joined to the drug at any of positions a, b, or c. As described in more detail below, such conjugates are conveniently prepared using a heterobifunctional

linker. In a preferred aspect of the invention, the heterobifunctional linker includes, on one end, a thiol acceptor (*e.g.*, a maleimide or haloacetamide group) for ligand conjugation and, at the other end, a hydrazide which forms an acylhydrazone linkage to a drug or drug analog that has carbonyl (aldehyde or ketone) functionality. Incorporation of a hydrazone into the conjugate is particularly preferred in order to impart desired pH-sensitivity to the conjugate. Thus, under low pH conditions, as in acidic intracellular compartments (*e.g.*, lysosomes), the hydrazone will cleave and release the drug free from the ligand. A preferred linker is an acid-labile linker, that is cleaved under low pH conditions, *i.e.*, conditions where the pH is less than about 6, preferably less than about 5.

10 The drugs of the present invention may be generally described as pentapeptides. Typically, pentapeptide drugs of the present invention can be prepared by introduction of a peptide bond between selected amino acids and/or modified amino acids, and/or peptide fragments, for example according to the liquid phase synthesis method (*see* E. Schröder and K. Lübke, "The Peptides", volume 1, pp 76-136, 1965, Academic Press) well known in the field of peptide chemistry.

15 A preferred method to prepare pentapeptide drugs of the present invention entails preparing a dipeptide and a tripeptide, and combining stoichiometric equivalents of these two fragments in a one-pot reaction with under suitable condensation conditions. This approach is illustrated in the following Schemes 1-3. Thus, the tripeptide **6** may be prepared as shown in Scheme 1, and the dipeptide **9** may be prepared as shown in Scheme 20 2. The two fragments **6** and **9** may be condensed to provide a pentapeptide as shown in Scheme 3.

Scheme 1



As illustrated in Scheme 1, a Z-protected amino acid 1 (where R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocyclic when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocyclic group of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6) is coupled to *t*-butyl ester 2 (where R⁶ is selected from hydrogen and lower alkyl; and R⁷ is *sec*-butyl or *iso*-butyl) under suitable coupling conditions, *e.g.*, in the

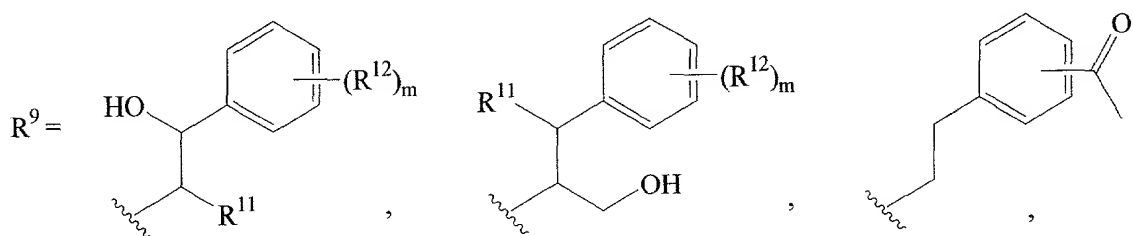
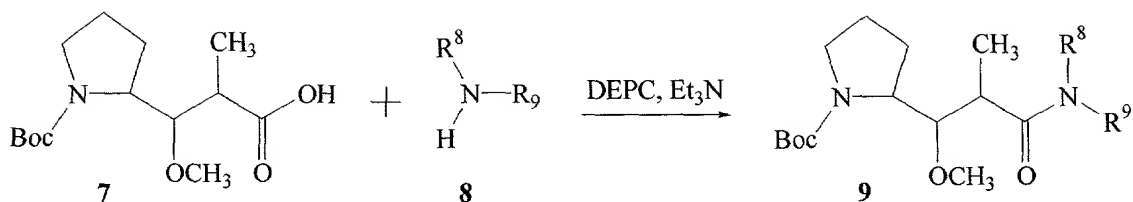
presence of PyBrop and diisopropylethylamine, or using DCC (as accomplished, for example, by Miyazaki, K. *et. al. Chem. Pharm. Bull.* 1995, 43(10), 1706-1718).

A preferred amino acid **1** is Z-Ile, while other suitable protected amino acids include, without limitation: Fmoc-cyclohexylglycine, Fmoc-cyclohexylalanine, Fmoc-aminocyclopropane-1-carboxylic acid, Fmoc- α -aminoisobutyric acid, Z-phenylalanine, 5 Fmoc-phenylglycine, and Fmoc-*tert*-butylglycine. A preferred *t*-butyl ester **2** is dolaisoleuine *t*-butyl ester.

After chromatographic purification, the dipeptide **3** is deprotected, *e.g.*, using H₂, 10% Pd-C in ethanol for Z-removal, or diethylamine for Fmoc-removal. The 10 resulting amine **4** readily forms a peptide bond with an amino acid **5** (where R¹ is selected from hydrogen and lower alkyl; R² is selected from hydrogen and lower alkyl; and R³ is lower alkyl). *N,N*-Dialkyl amino acids are preferred amino acids **5**, such as commercially available *N,N*-dimethyl valine. Other *N,N*-dialkyl amino acids can be prepared by reductive bis-alkylation following known procedures (*see, e.g.*, Bowman, R.E, Stroud, H.H 15 *J. Chem. Soc.*, 1950, 1342-1340). Fmoc-Me-L-Val and Fmoc-Me-L-glycine are two preferred amino acids **5** for the synthesis of *N*-monoalkyl derivatives. The amine **4** and the amino acid **5** are conveniently joined to provide the tripeptide **6** using coupling reagent DEPC with triethylamine as the base.

The dipeptide **9** can be readily prepared by condensation of the modified 20 amino acid *t*-Boc-Dolaproine **7** (*see*, for example, Pettit, G.R., *et al. Synthesis*, 1996, 719-725), with commercially available (1*S*, 2*R*)-norephedrine, L- or D-phenylalaninol, or with synthetic *p*-acetylphenethylamine **8** (Shavel, J., Bobowski, G., 1969, U.S. Patent 3,445,518) using condensing agents well known for peptide chemistry, such as, for example, DEPC in the presence of triethylamine, as shown in Scheme 2.

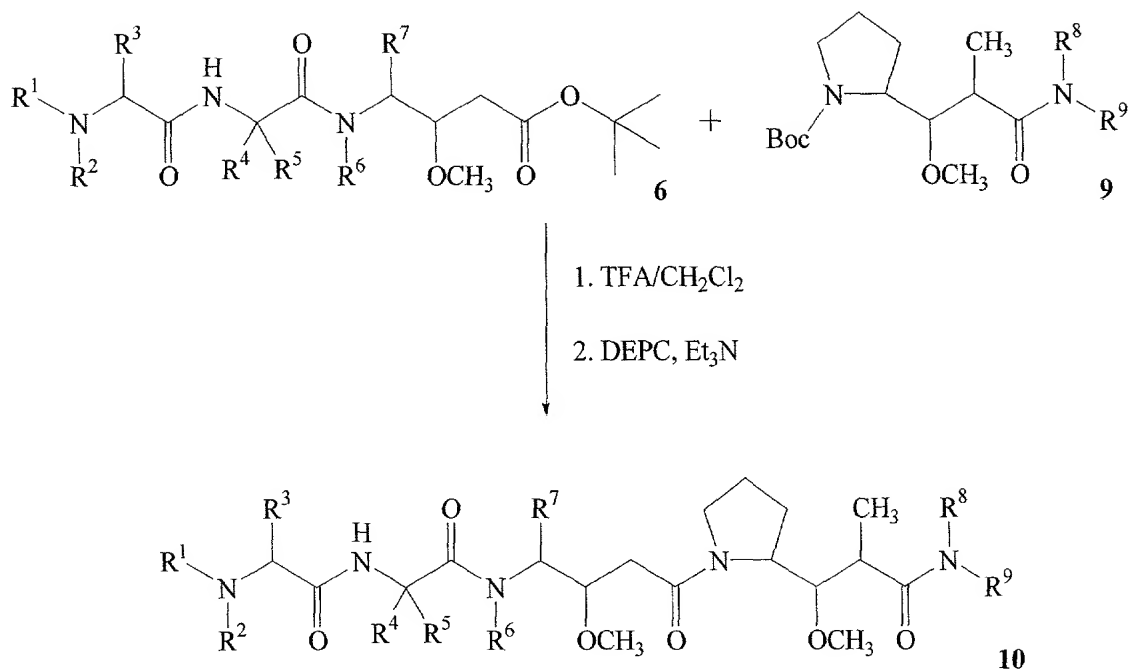
Scheme 2



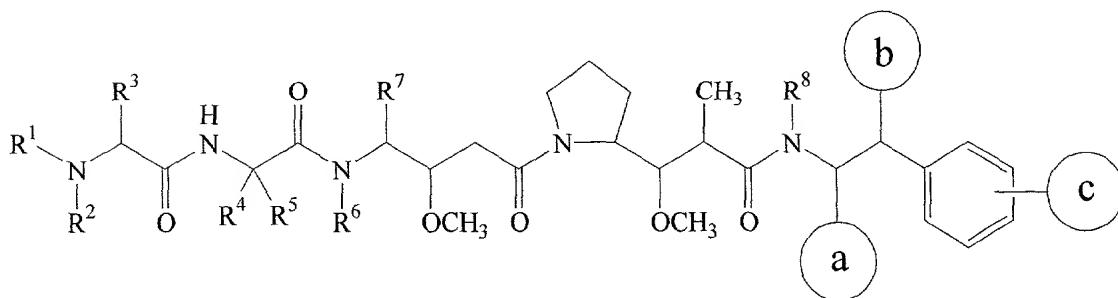
Scheme 3 illustrates the joining together of the tripeptide **6** with the dipeptide **9** to form a pentapeptide **10**. The joining together of these two fragments can be accomplished using, *e.g.*, TFA to facilitate Boc and *t*-butyl ester cleavage, respectively, followed by condensation conditions, *e.g.*, utilizing DEPC, or similar coupling reagent, in the presence of excess base (triethylamine or equivalent) to give the desired product in moderate but acceptable yields.

To obtain *N*-monoalkyl pentapeptide **10**, the Fmoc group should be removed from the N-terminal amino acid after final coupling by treatment with diethylamine (see General Procedure E described below).

Scheme 3



The pentapeptides of the structure

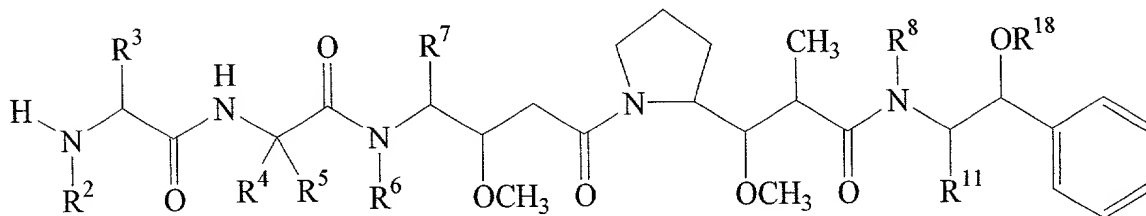


5

are drugs of the present invention, upon appropriate selection of a, b, and c, and may be prepared as described above. Thus, as referred to herein, the drugs of the present invention are not necessarily the same as the degradation product of the prodrugs of the present invention. Rather, the drugs of the present invention are pharmacologically active, cytotoxic chemicals of the above structure, which are conveniently utilized to prepare prodrugs of the present invention.

10

Thus, in one aspect, the drug of the present invention has the formula



wherein, independently at each location:

5 R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R^6 is selected from hydrogen and lower alkyl;

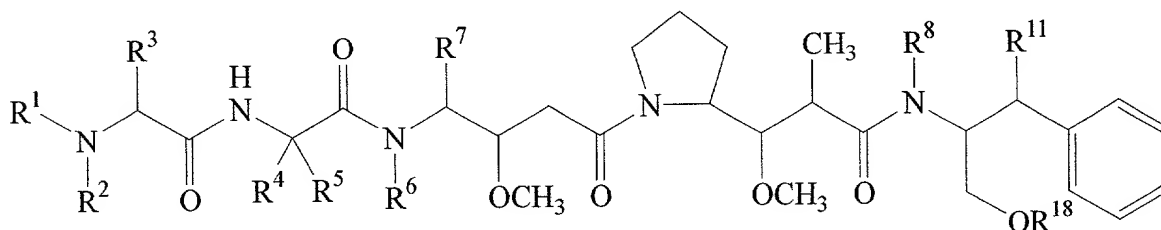
R^7 is *sec*-butyl or *iso*-butyl;

R^8 is selected from hydrogen and lower alkyl;

R^{11} is selected from hydrogen and lower alkyl; and

15 R^{18} is selected from hydrogen, a hydroxyl protecting group, and a direct bond where OR^{18} represents =O.

In another aspect, the drug of the invention has the structure



20 wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

R^2 is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R⁶ is selected from hydrogen and lower alkyl;

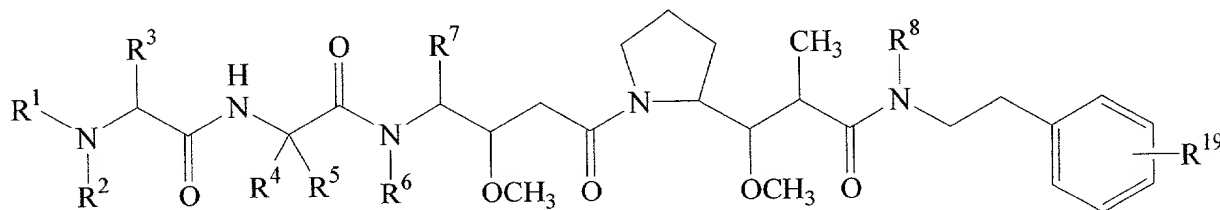
R⁷ is *sec*-butyl or *iso*-butyl;

R⁸ is selected from hydrogen and lower alkyl;

R¹¹ is selected from hydrogen and lower alkyl; and

R¹⁸ is selected from hydrogen, a hydroxyl protecting group, and a direct bond where OR¹⁸ represents =O.

In another aspect, the drug of the invention has the structure



wherein, independently at each location:

R¹ is selected from hydrogen and lower alkyl;

R² is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

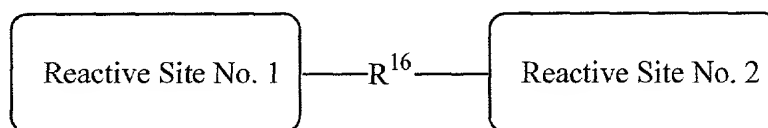
R⁶ is selected from hydrogen and lower alkyl;

R⁷ is *sec*-butyl or *iso*-butyl;

R⁸ is selected from hydrogen and lower alkyl; and

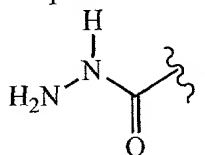
R¹⁹ is selected from hydroxy- and oxo-substituted lower alkyl.

In order to prepare a prodrug of the present invention, the drug is directly reacted with a linker, typically a heterobifunctional linker, or else the drug is somewhat modified in order that it contains a reactive site that is suitably reactive with a reactive site on a heterobifunctional linker. In general, the heterobifunctional linker of the present invention has the structure



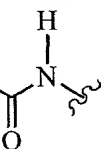
In a preferred embodiment of the invention, Reactive Site No. 1 is reactive with a carbonyl group of the drug, Reactive Site No. 2 is reactive with functionality on the ligand, and Reactive Sites 1 and 2 are reactive with different functional groups.

Many suitable heterobifunctional linkers are known in the art. *See, e.g.,* S. S. Wong, *Chemistry of Protein Conjugation and Crosslinking*, CRC Press Inc., Boston 1991. In one aspect of the invention, Reactive Site No. 1 is a hydrazide, (*i.e.*, a group of the formula

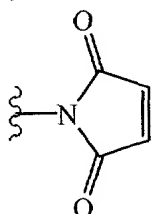


thiol-accepting group. Suitable thiol-accepting groups include haloacetamide groups (*i.e.*,

groups of the formula $X-CH_2-C(=O)-NH-$ where X represents a halide) and maleimide groups



(*i.e.*, a group of the formula maleimide). In the heterobifunctional linker of the present



invention, R^{16} is selected from divalent lower alkyl, divalent aryl, and $-(CH_2OCH_2)_pCH_2-$ where p is 1-5.

In general, suitable heterobifunctional linkers include commercially available maleimido hydrazides (*e.g.*, β -maleimido propionic acid hydrazide, e-

maleimidocaproic acid hydrazide, and SMCC hydrazide, available from Molecular Biosciences, Inc. Boulder CO). Alternatively, the heterobifunctional linker can be easily prepared from a maleimido acid, including polyoxoethylene-based maleimido acids (Frisch, B., *et al.*, *Bioconjugate Chem.*, 1996, 7, 180-186) according to known procedures (King, H.D., *et al.*, *Bioconjugate Chem.*, 1999, 10, 279-288). According to this procedure, maleimido acids are first converted to their *N*-hydroxysuccinimide esters, followed by reaction with *tert*-butylcarbazate. After purification and Boc removal the maleimido hydrazide are usually obtained in good yields. Haloacetamide hydrazide heterobifunctional linkers can be prepared from available intermediates, for example succinimidyl 3-(bromoacetamido)propionate, or [2-[2-(2-bromo-acetyl-amino)-ethoxy]-ethoxy]-acetic acid (Frisch, B., *et al.*, *Bioconjugate Chem.*, 1996, 7, 180-186) by the reaction with *tert*-butylcarbazate described above.

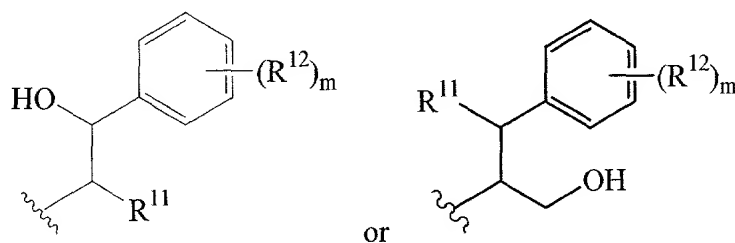
In another aspect of the invention, Reactive Site No. 2 is an amine-reactive group. Suitable amine reactive groups include activated esters such as *N*-hydroxysuccinimide esters, *p*-nitrophenyl esters, pentafluorophenyl esters, and other reactive functionalities such as isothiocyanates, isocyanates, anhydrides, acid chlorides, and sulfonyl chlorides.

As indicated previously, in a preferred embodiment, the synthesis of the drug should provide for the presence of a carbonyl group, or at least a group that can be readily converted to or elaborated to form a carbonyl group. A carbonyl group may be readily made part of, or added to, the pentapeptide drug by any of the following exemplary procedures:

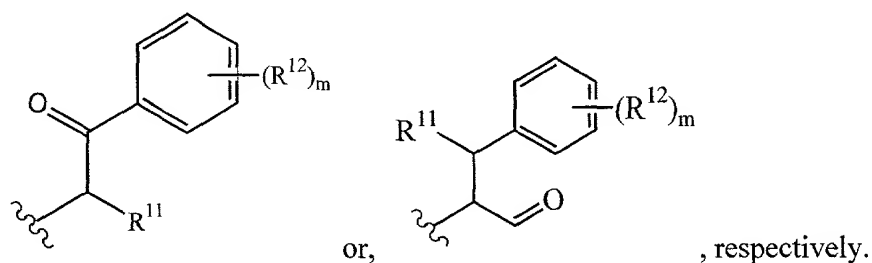
- (1) oxidation of a hydroxyl group of the drug to form a carbonyl;
- (2) reaction of a hydroxyl group of the drug with a molecule that contains both a carbonyl or protected carbonyl and a hydroxyl-reactive group, *e.g.*, a carboxylic acid or ester. For instance, the hydroxyl group of the drug may be reacted with a keto acid or a keto ester, so as to convert the hydroxyl group to an ester group, where the ester group is joined to the keto group of the keto acid/ester;

(3) introducing a carbonyl group to the drug during synthesis of the drug, *e.g.*, during peptide synthesis using carbonyl-containing amino acids, amines or peptides.

Thus, if the pentapeptide **10** includes a carbonyl group, then it can be
 5 reacted directly with the heterobifunctional linker containing a carbonyl-reactive end, *e.g.*, a hydrazide group. If the pentapeptide **10** does not include a carbonyl group, then a carbonyl group may be introduced into the molecule in order to impart hydrazide reactivity to the drug. Thus, when R^9 is either of



10 then the pentapeptide **10** can be subjected to oxidizing conditions, *e.g.*, pyridinium chlorochromate (PCC)/pyridine (*see, e.g., Synthesis*, 1982, 245, 881, review), in order to provide the corresponding oxidized compounds wherein R^9 is



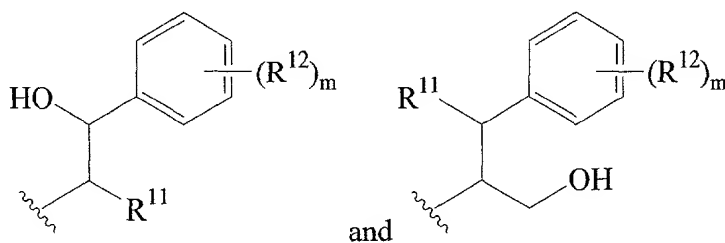
Alternatively, if the pentapeptide **10** does not contain a carbonyl group but
 15 does contain a hydroxyl group, then this hydroxyl group can be elaborated to provide a carbonyl group. One way to provide carbonyl functionality from hydroxyl functionality is to react the hydroxyl group with a keto acid. The chemical literature describes many keto acids, and some keto acids are also available from commercial sources. Most keto acids, although not α and β aliphatic keto acids, can be readily condensed with a hydroxyl group
 20 using DCC/DMAP chemistry to provide keto esters **11** (Larock, R.C., *Comprehensive Organic Transformations*, Wiley-VCH, 1999, p. 1937). Exemplary keto acids include,

without limitation: levulinic acid, 4-acetylbutyric acid, 7-oxooctanoic acid, 4-acetylbenzoic acid, 4-carboxyphenylacetone, *N*-methyl-4-acetyl-3,5-dimethyl-2-pyrrole carboxylic acid, 3-benzoyl-2-pyridinecarboxylic acid, and 2-acetylthiazol-4-carboxylic acid.

a-Keto acids require initial protection of the carbonyl group as, for example, a dimethyl ketal. Dimethyl ketals can be readily obtained by treatment of keto acids with an excess of dimethylorthoformate in the presence of concentrated sulfuric acid as described in LaMattina, J.L., Muse, D.E. *J. Org. Chem.* 1987, 52, 3479-3481. After protection, standard DCC/DMAP-mediated condensation of the carboxyl group of the keto acid with the hydroxyl group of the pentapeptide can be used to form the ester bond. After aqueous work-up and low vacuum drying, this material may be used without further purification. Once the ester bond is formed, the dimethyl ketal can be hydrolyzed under mild acidic conditions to give the desired a-keto ester **11**. Exemplary a-keto acids include, without limitation: pyruvic acid, 2-ketobutyric acid, 2-thiophenglyoxylic acid, and benzoylformic acid.

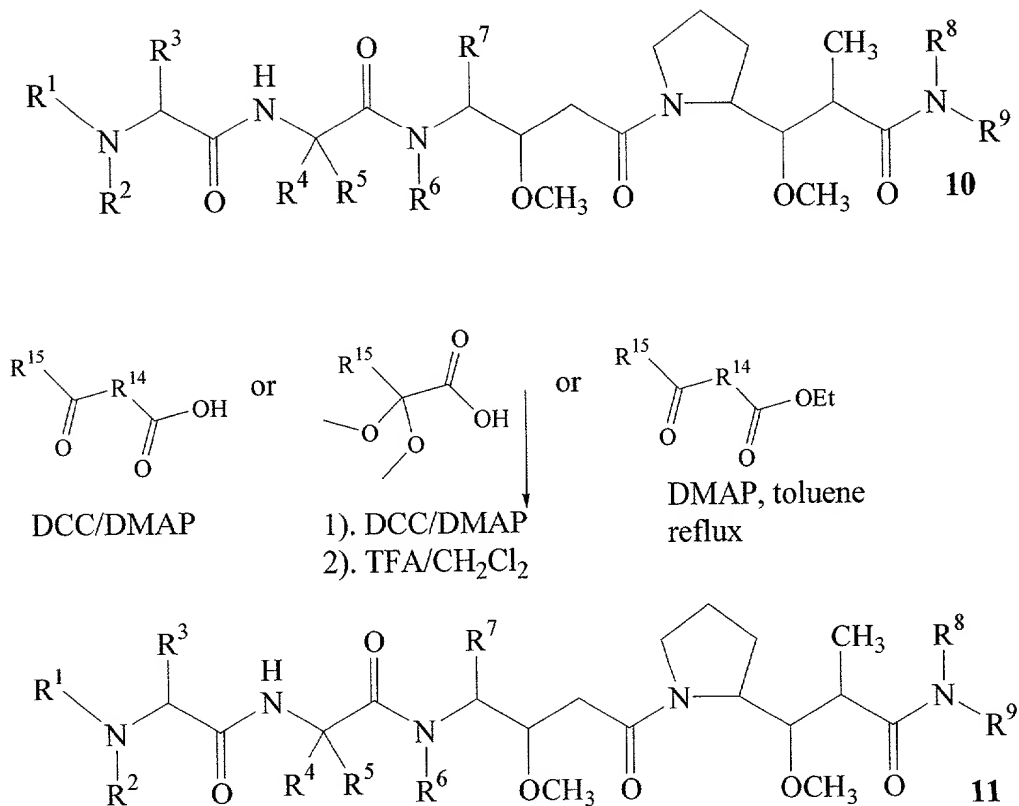
As illustrated in Scheme 4, preparation of β -keto esters of pentapeptides may be performed via DMAP-promoted transesterification of ethyl β -keto esters (Otera, J. *Chem. Rev.*, 1993, 93, 1449-1470). Commercially available β -keto esters that may be used in this transformation include, without limitation, ethyl acetoacetate, ethyl β -oxo-3-furanpropionate, and ethyl 3-(1-adamantyl)-3-oxopropionate.

For instance, when R^9 is selected from

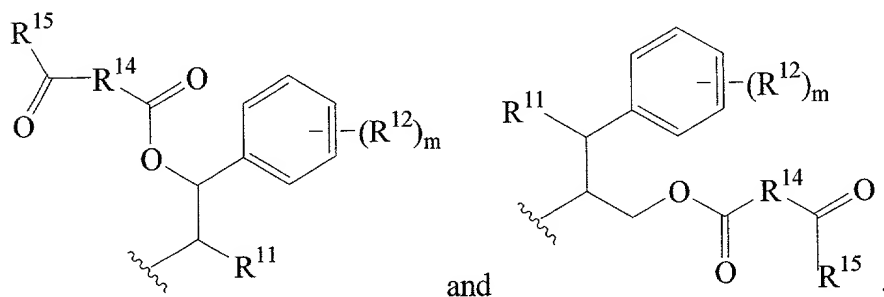


the hydroxyl group of R^9 may be reacted with a ketoacid to form an ester linkage and the desired carbonyl group. This approach to preparing carbonyl-containing drugs is shown in Scheme 4.

Scheme 4



where, in compound 11, R⁹ is selected from

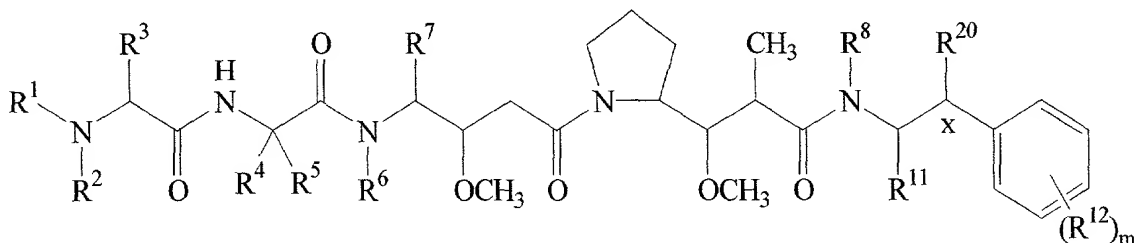


Once formed, the keto esters of pentapeptides can be isolated from the reaction mixture and purified by, for example, flash silica gel chromatography, and/or reversed phase high performance liquid chromatography.

After reaction with a heterobifunctional linker, the resulting product is a
10 pentapeptide drug conjugated to a linker moiety, where the linker moiety is a reactive

linker moiety. In other words, the pentapeptide-reactive linker contains a group that is reactive with functionality present on the ligand.

Thus, in one aspect, the present invention provides a compound of the formula



5

wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

R^2 is selected from hydrogen and lower alkyl;

10

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

15

R^6 is selected from hydrogen and lower alkyl;

R^7 is *sec*-butyl or *iso*-butyl;

R^8 is selected from hydrogen and lower alkyl;

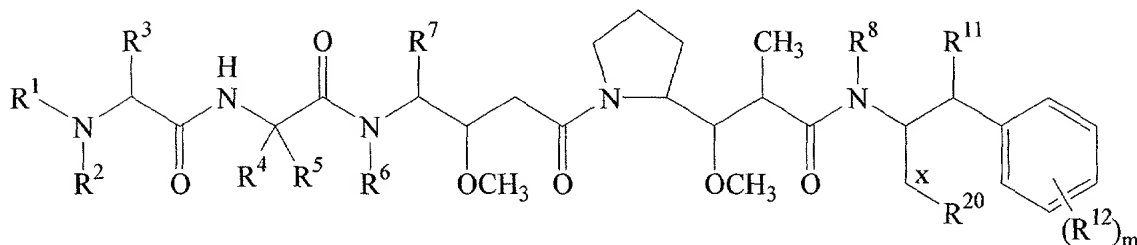
R^{11} is selected from hydrogen and lower alkyl;

R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where

20 R^{12} is independently selected at each occurrence; and

R^{20} is a reactive linker group having a reactive site that allows R^{20} to be reacted with a targeting moiety.

In another aspect, the present invention provides a compound of the formula



wherein, independently at each location:

5 R¹ is selected from hydrogen and lower alkyl;

R² is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocycle when R⁵ is
 10 selected from H and methyl, or R⁴ and R⁵ together form a carbocycle of the partial formula
 -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl
 and n is selected from 2, 3, 4, 5 and 6;

R⁶ is selected from hydrogen and lower alkyl;

R⁷ is *sec*-butyl or *iso*-butyl;

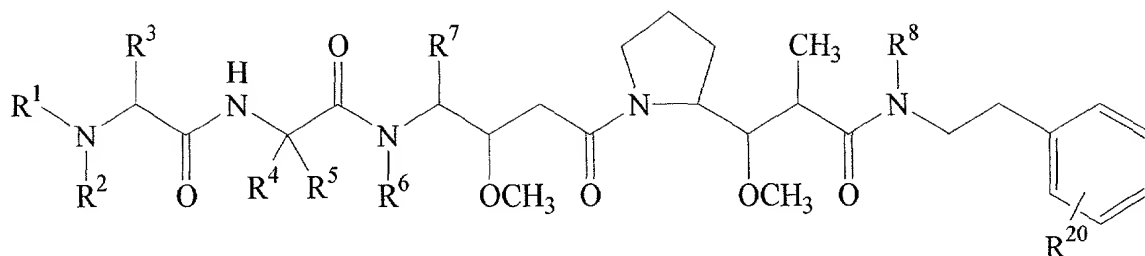
R⁸ is selected from hydrogen and lower alkyl;

15 R¹¹ is selected from hydrogen and lower alkyl;

R¹² is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where
 R¹² is independently selected at each occurrence; and

R²⁰ is a reactive linker group having a reactive site that allows R²⁰ to be
 reacted with a targeting moiety.

20 In yet another aspect, the present invention provides a compound of the
 formula



wherein, independently at each location:

R^1 is selected from hydrogen and lower alkyl;

5 R^2 is selected from hydrogen and lower alkyl;

R^3 is lower alkyl;

R^4 is selected from lower alkyl, aryl, and $-\text{CH}_2-\text{C}_{5-7}\text{carbocycle}$ when R^5 is selected from H and methyl, or R^4 and R^5 together form a carbocycle of the partial formula $-(\text{CR}^a\text{R}^b)_n-$ wherein R^a and R^b are independently selected from hydrogen and lower alkyl
10 and n is selected from 2, 3, 4, 5 and 6;

R^6 is selected from hydrogen and lower alkyl;

R^7 is *sec*-butyl or *iso*-butyl;

R^8 is selected from hydrogen and lower alkyl; and

R^{20} is a reactive linker group having a reactive site that allows R^{20} to be
15 reacted with a targeting moiety.

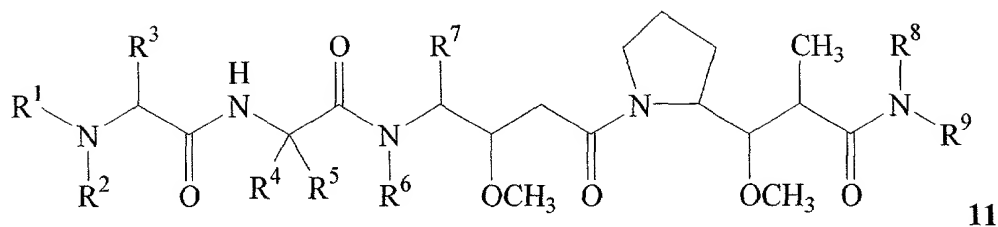
As shown in Scheme 5, hydrazone bond formation between the heterobifunctional linker and the carbonyl-containing pentapeptide drug, followed by treatment with the ligand, will provide compounds of formula I. The reaction of the drug with the heterobifunctional linker may be performed in a suitable solvent, *e.g.*, anhydrous
20 MeOH or DMSO, preferably in the presence of a small amount of acid, *e.g.*, 0.1 % AcOH or TFA at room temperature. The relative amounts of hydrazide to be applied and the time required for complete, or near complete reaction depends on the nature of the carbonyl group. Aromatic and α -carbonyl groups generally require higher excess of hydrazide and longer reaction time. The procedures of King, H. D., *et al. Bioconjugate Chem.*, 1999, 10,
25 279-288, as used to prepare Doxorubicin-mAb conjugates, may be utilized to prepare the

conjugates of the present invention. Stable hydrazones may be purified by a reversed phase HPLC. Hydrazone stability typically depends on the nature of corresponding keto esters.

The ligand is preferably a targeting ligand that directs the conjugate to a
5 desired location. A preferred targeting ligand is an antibody, *e.g.*, a chimeric or humanized antibody, or a fragment thereof. Internalizing monoclonal antibodies: BR96, AC10, herceptin, S2C6 are preferred antibodies of the present invention. The ligand is suitably joined to the linker through a free thiol group or a free amino group on the ligand. For instance, when the ligand is an antibody, the free thiol group may be generated by
10 reduction of a disulfide group present in the antibody with dithiothreitol as previously described (Trail, P. A. et al., *Science* 1993, 261, 212-215, and Firestone, R. A. Willner, D., Hofstead, S. J., King, H. D., Kaneko, T, Braslawsky, G. R., Greenfield, R. S., Trail, P. A., Lasch, S. J., Henderson, A. J., Casazza, A. M., Hellström, I., and Hellström, K. E., "Synthesis and antitumor activity of the immunoconjugate BR96-Dox" *J. Controlled Rel.*
15 1996, 39, 251-259). Alternatively, a lysine of the antibody may be reacted with iminothiolane hydrochloride (Traut's reagent) to introduce free thiol groups. Alternatively, free amino groups of the antibody may be reacted directly with linker-drug bearing activated ester, isothiocyanate, isocyanate, anhydride, or acyl chloride functionalities. The conjugate of the invention retains both specificity and therapeutic drug activity for the
20 treatment of cell population expressing specific antigen for the ligand. Stable in serum, the hydrazone bond of the conjugate will be cleaved in acidic intracellular compartments releasing free highly-potent drug. This approach provides conjugates with up to 1000 fold selectivity between target and non-target cell lines.

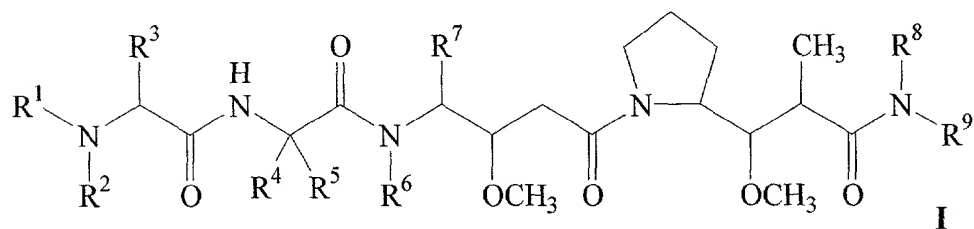
Scheme 5 illustrates the conjugation of the carbonyl-containing drug to a
25 ligand through a heterobifunctional linker to form a compound of formula I.

Scheme 5



1. Heterobifunctional Linker

2. HS-mAb, or
NH₂-mAb



wherein, in the compound of formula I, and independently at each location;

R¹ is selected from hydrogen and lower alkyl;

R² is selected from hydrogen and lower alkyl;

R³ is lower alkyl;

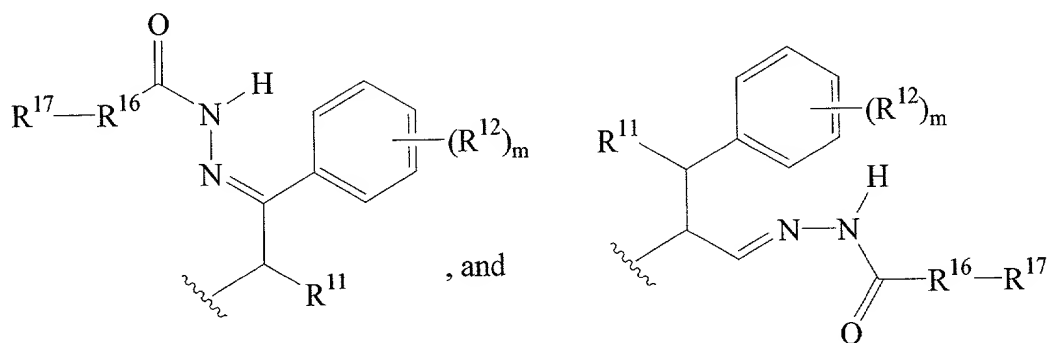
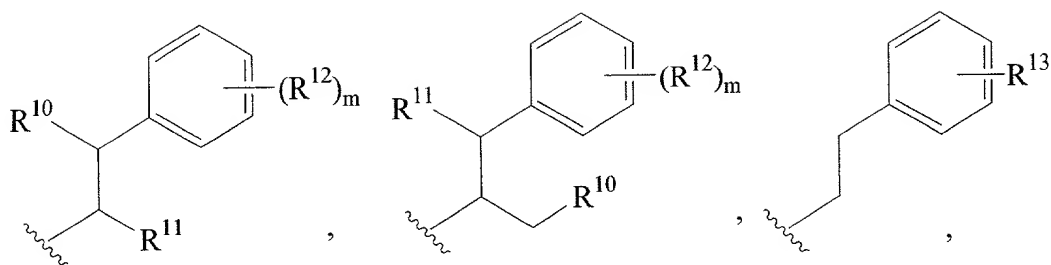
R⁴ is selected from lower alkyl, aryl, and -CH₂-C₅₋₇carbocyclic when R⁵ is selected from H and methyl, or R⁴ and R⁵ together form a carbocyclic group of the partial formula -(CR^aR^b)_n- wherein R^a and R^b are independently selected from hydrogen and lower alkyl and n is selected from 2, 3, 4, 5 and 6;

R⁶ is selected from hydrogen and lower alkyl;

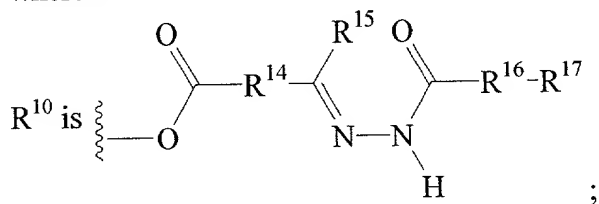
R⁷ is *sec*-butyl or *iso*-butyl;

R⁸ is selected from hydrogen and lower alkyl;

R⁹ is selected from

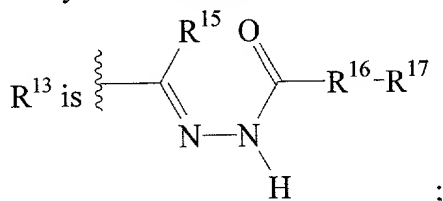


wherein



R^{11} is selected from hydrogen and lower alkyl;

5 R^{12} is selected from lower alkyl, halogen, and methoxy, and m is 0-5 where R^{12} is independently selected at each occurrence;

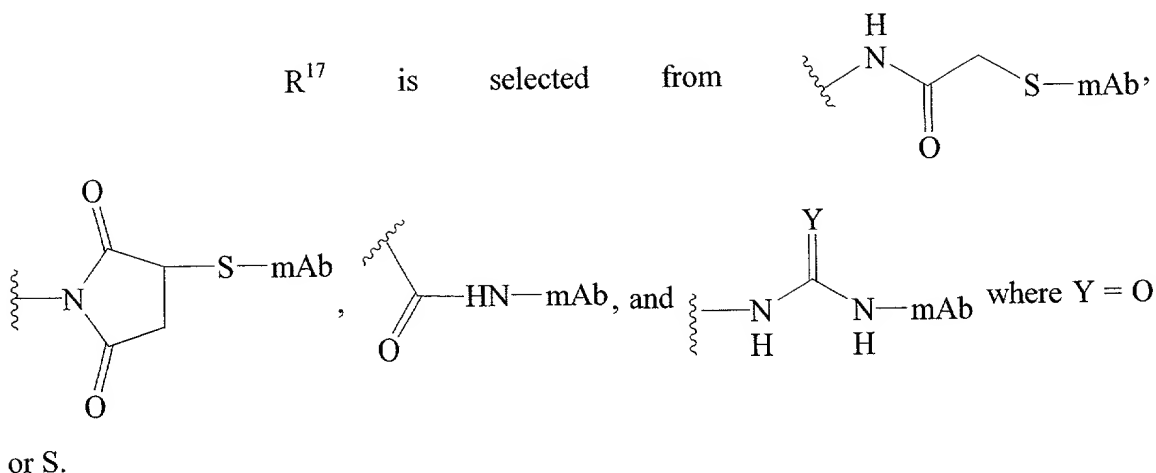


wherein:

10 R^{14} is selected from a direct bond, divalent lower alkyl and divalent aryl;

R^{15} is selected from hydrogen, lower alkyl and aryl;

R^{16} is selected from divalent lower alkyl, divalent aryl, and $-(CH_2OCH_2)_pCH_2-$ where p is 1-5; and



- 5 As indicated by the identity of R^{17} , a preferred ligand is a monoclonal-antibody as defined herein. Some preferred monoclonal antibodies include BR96 mAb (Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S. J., Casazza, A. M., Firestone, R. A., Hellström, I., Hellström, K. E., "Cure of Xenografted Human Carcinomas by BR96-Doxorubicin Immunoconjugates" *Science* 1993, 261, 212-215), BR64 (Trail, PA,
- 10 Willner, D, Knipe, J., Henderson, A. J., Lasch, S. J., Zoeckler, M. E., Trailsmith, M. D., Doyle, T. W., King, H. D., Casazza, A. M., Braslawsky, G. R., Brown, J. P., Hofstead, S. J., Greenfield, R. S., Firestone, R. A., Mosure, K., Kadow, D. F., Yang, M. B., Hellstrom, K. E., and Hellstrom, I. "Effect of Linker Variation on the Stability, Potency, and Efficacy of Carcinoma-reactive BR64-Doxorubicin Immunoconjugates" *Cancer Research* 1997, 57,
- 15 100-105, herceptin (Stebbing, J., Copson, E., and O'Reilly, S. "Herceptin (trastuzumab) in advanced breast cancer" *Cancer Treat Rev.* 26, 287-90, 2000), mAbs against the CD 40 antigen, such as S2C6 mAb (Francisco, J. A., Donaldson, K. L., Chace, D., Siegall, C. B., and Wahl, A. F. "Agonistic properties and in vivo antitumor activity of the anti-CD-40 antibody, SGN-14" *Cancer Res.* 2000, 60, 3225-3231), and mAbs against the CD30
- 20 antigen, such as AC10 (Bowen, M. A., Olsen, K. J., Cheng, L., Avila, D., and Podack, E. R. "Functional effects of CD30 on a large granular lymphoma cell line YT" *J. Immunol.*, 151, 5896-5906, 1993). Many other internalizing mAbs that bind to tumor associated antigens can be used in this invention, and have been reviewed (Franke, A. E., Sievers, E. L., and Scheinberg, D. A., "Cell surface receptor-targeted therapy of acute myeloid

leukemia: a review" *Cancer Biother Radiopharm.* 2000,15, 459-76; Murray, J. L., "Monoclonal antibody treatment of solid tumors: a coming of age" *Semin Oncol.* 2000, 27, 64-70; Breitling, F., and Dubel, S., *Recombinant Antibodies*, John Wiley, and Sons, New York, 1998).

5 Compositions

In other aspects, the present invention provides pentapeptide drugs as described above, and prodrugs as also described above that are based on the pentapeptide drugs, in combination with a pharmaceutically acceptable carrier, excipient or diluent. For convenience, the pentapeptide drugs and the prodrugs of the invention will simply be referred to as compounds of the invention. Thus, the present invention provides a pharmaceutical or veterinary composition (hereinafter, simply referred to as a pharmaceutical composition) containing a compound of the invention as described above, in admixture with a pharmaceutically acceptable carrier. The invention further provides a composition, preferably a pharmaceutical composition, containing an effective amount of a compound as described above, in association with a pharmaceutically acceptable carrier.

The pharmaceutical compositions of the present invention may be in any form that allows for the composition to be administered to an animal subject. For example, the composition may be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral, sublingual, rectal, vaginal, ocular, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Pharmaceutical compositions of the invention are formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to an animal subject. Compositions that will be administered to a subject take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of the invention in aerosol form may hold a plurality of dosage units.

Materials used in preparing the pharmaceutical compositions should be pharmaceutically pure and non-toxic in the amounts used. It will be evident to those of ordinary skill in the art that the optimal dosage of the active ingredient(s) in the pharmaceutical composition will depend on a variety of factors. Relevant factors include, without limitation, the type of subject (*e.g.*, human), the particular form of the active ingredient, the manner of administration, and the composition employed.

In general, the pharmaceutical composition includes an (where "a" and "an" refers here, and throughout this specification, as one or more) active compounds of the invention in admixture with one or more carriers. The carrier(s) may be particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) may be liquid, with the compositions being, for example, an oral syrup or injectable liquid. In addition, the carrier(s) may be gaseous, so as to provide an aerosol composition useful in, *e.g.*, inhalatory administration.

When intended for oral administration, the composition is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following adjuvants may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrans, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin, a flavoring agent such as peppermint, methyl salicylate or orange flavoring, and a coloring agent.

When the composition is in the form of a capsule, *e.g.*, a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrin or a fatty oil.

The composition may be in the form of a liquid, *e.g.*, an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred composition contain, in addition to the present compounds, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition for administration by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

The liquid pharmaceutical compositions of the invention, whether they are solutions, suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, cyclodextrin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

A liquid composition intended for either parenteral or oral administration should contain an amount of a compound of the present invention such that a suitable dosage will be obtained. Typically, this amount is at least 0.01% of a compound of the invention in the composition. When intended for oral administration, this amount may be varied to be between 0.1% and about 80% of the weight of the composition. Preferred oral compositions contain between about 4% and about 50% of the compound of the invention. Preferred compositions and preparations according to the present invention are prepared so that a parenteral dosage unit contains between 0.01% to 2% by weight of active compound.

The pharmaceutical composition may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base.

The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, beeswax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device. Topical formulations may contain a concentration of a compound of the present invention of from about 0.1% to about 10% w/v (weight per unit volume).

The composition may be intended for rectal administration, in the form, *e.g.*, of a suppository which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.

The composition may include various materials that modify the physical form of a solid or liquid dosage unit. For example, the composition may include materials that form a coating shell around the active ingredients. The materials that form the coating shell are typically inert, and may be selected from, for example, sugar, shellac, and other enteric coating agents. Alternatively, the active ingredients may be encased in a gelatin capsule.

The pharmaceutical composition of the present invention may consist of gaseous dosage units, *e.g.*, it may be in the form of an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of compounds of the invention may be delivered in single phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, spacers and the like, which together may form a kit. Preferred aerosols may be determined by one skilled in the art, without undue experimentation.

Whether in solid, liquid or gaseous form, the pharmaceutical composition of the present invention may contain one or more known pharmacological agents used in the treatment of cancer.

The pharmaceutical compositions may be prepared by methodology well known in the pharmaceutical art. For example, a composition intended to be administered by injection can be prepared by combining a compound of the invention with water so as to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with a compound of the invention so as to facilitate dissolution or homogeneous suspension of the active compound in the aqueous delivery system.

Biological Activity and Utility of Compounds

The present invention provides biologically-active compounds and pro-drugs (collectively, compounds, or compounds of the invention), methods of preparing compounds of the invention, pharmaceutical compositions comprising the compounds of the invention, and methods for treatment of cancers and other tumors in animal subjects. For instance, the invention provides compounds and compositions for use in a method for treating tumors wherein the animal subject is treated, in a pharmaceutically acceptable manner, with a pharmaceutically effective amount of a compound or composition of the present invention.

The compounds and compositions of the invention can be used in a variety of settings for the treatment of mammalian cancers. The mAb-pentapeptide conjugates can be used to deliver the cytotoxic drug to tumor cells. Once the antibody has bound to tumor associated antigens, it is taken up inside cells through receptor-mediated endocytosis into endosomes and lysosomes. These intracellular vesicles are acidic and can induce the hydrolysis of an acid-sensitive linker bond, *e.g.*, a hydrazone bond, between the drugs and the mAbs. In addition, ester bonds can be cleaved by proteases and esterases, which are in abundance within lysosomes. The released drug is then free to migrate in the cytosol and induce cytotoxic activities.

The specificity of the mAb for a particular tumor type will dictate which tumors will be treated with the immunoconjugates. For example, BR96-pentapeptide conjugates will be used to treat antigen positive carcinomas including those of the lung, breast, colon, ovaries, and pancreas. Anti-CD30-pentapeptide and anti-CD40-pentapeptide conjugates may be used for treating hematologic malignancies.

The free drugs of the invention may also be used as chemotherapeutics in the untargeted form. For example, the pentapeptides of the present invention may be used against ovarian, CNS, renal, lung, colon, melanoma, and hematologic tumors.

Conjugation of these highly potent drugs to monoclonal antibodies specific for tumor markers results in specific targeting, thus reducing general toxicity of these compounds. pH-Sensitive linkers should provide stability of the conjugate in blood, yet should be hydrolyzed in acidic intracellular compartments liberating active drug.

The following examples are provided by way of illustration and not limitation.

EXAMPLES

The following examples may make reference to general procedures as set forth below. In addition, Figure 1 illustrates the preparation of specific tripeptides, while Figure 2 illustrates the preparation of specific dipeptides useful in preparing compounds of the present invention. Figures 3, 4 and 5 illustrate the preparation of specific pentapeptides. Figures 6, 7, 8 and 9 illustrate the preparation of specific drugs of the invention having exemplary reactive linkers. Figures 10 and 11 illustrate the preparation of mAb-drug conjugates.

Coupling procedure A: Peptide synthesis using DEPC

To a cooled (ice bath) solution of [2S-[2R*(α S*, β S*)]]-1-[(1,1-dimethylethoxy) carbonyl]- β -methoxy- α -methyl-2-pyrrolidinepropanoic acid (*t*-Boc-dolaproine, **20**) 0.27 g, 0.94 mmol) in anhydrous CH₂Cl₂ (10 mL) is added an identified amine (1.03 mmol, 1.1 eq.) followed by triethylamine (0.40 mL, 2.8 mmol, 3.0 eq.) and DEPC (0.17 mL, 90 %, 1.03 mmol, 1.1 eq.). The resulting solution is stirred under argon

for 12 hours. The solvent is removed under reduced pressure at room temperature, and the residue is chromatographed (silica gel column using 4:1 hexanes-acetone as eluent). After the evaporation of solvent from the fractions selected according to TLC analysis, the residue is dried under vacuum overnight to afford the amide.

5 Coupling procedure B: Peptide synthesis using PyBrop

The amino acid (1.0 eq.) containing a carboxyl protecting group, if necessary, was dissolved in anhydrous CH_2Cl_2 followed by the addition of diisopropylethylamine (1.5 eq.). Fmoc-, Z-, or dimethyl amino acid/peptide (1.1 eq.) was added as a solid in one portion and the dissolved mixture had added PyBrop (1.2 eq.). The reaction was monitored by TLC or HPLC.

General procedure C: Pentapeptide synthesis

A solution of dipeptide and tripeptide (1 eq. each) in CH_2Cl_2 (10 mL) and trifluoroacetic acid (5 mL) is stirred (ice bath under a N_2 atmosphere) for two hours. The reaction may be monitored by TLC or, preferably, HPLC. The solvent is removed under reduced pressure and the residue dissolved in toluene. Solvent is again removed *in vacuo* and the process repeated. The residue is dried under high vacuum for 12 h and then dissolved in dry CH_2Cl_2 followed by the addition of diisopropylethylamine (1.5 eq.). Depending on the residues, the peptides may be coupled using either PyBrop or DEPC (1.2 eq.). The reaction mixture is monitored by either TLC or HPLC.

20 General procedure D: Z-removal via hydrogenolysis

Using a large, heavy-walled flask, a solution of Z-protected amino acid or peptide was dissolved in ethanol. 10% palladium on carbon was added (1% w/w peptide) and the mixture was introduced to H_2 . Reaction progress was monitored by HPLC and typically found to be complete within 1-2 h. The flask contents are filtered through a pre-washed pad of celite and then the celite is washed with methanol. The eluent solution is evaporated to an oil, dissolved in toluene, and re-evaporated.

General procedure E: Fmoc-removal using diethylamine

The Fmoc-containing compound is dissolved in CH_2Cl_2 to which an equal amount of diethylamine is added. Reaction progress is monitored by TLC (or HPLC) and is usually complete within 2 h. Solvents are removed *in vacuo* and the residue taken up in
5 toluene and concentrated again. The residue is dried under high vacuum for at least 1 h.

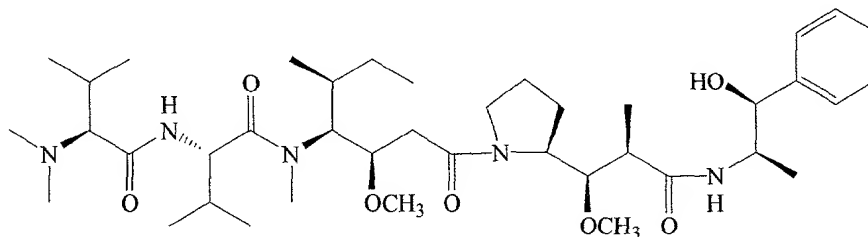
General procedure F: Hydrazone formation

Hydrazone formation is performed in anhydrous MeOH, 0.1 % AcOH (typically 1 mL per 10 mg of a drug) at room temperature. Time of the reaction (6 h - 5 days) and an amount of a hydrazide (2 – 30 eq.) may be varied depending on the specific
10 ketone. The reaction progress is monitored by C_{18} RP-HPLC. Typically, a newly formed acylhydrazone has lower retention time compared to the parent ketone. After the reaction is complete, DMSO (1 - 2 mL) is added to the reaction mixture and methanol is removed under reduced pressure. The residue is directly loaded onto a C_{18} RP column for preparative HPLC purification (Varian Dynamax column, 5 μ , 100 Å, linear gradient of
15 MeCN in 100 mM TEAA buffer, pH 7.0, from 10 to 95 % at flow rate 4 mL/min). The appropriate fractions are concentrated under reduced pressure, co-evaporated with acetonitrile (4×25 mL), and finally dried in deep vacuum for 2 days.

EXAMPLE 1

20

PREPARATION OF COMPOUND 29a



Preparation of Compound 14a

Compound **14a** was prepared by reacting compounds **12** and **13a** according to coupling procedure B. After concentration of the reaction mixture, the residue was directly injected onto a reverse phase preparative-HPLC column (Varian Dynamax column 5 21.4 mm x 25 cm, 5 μ , 100 Å, using an isocratic run of 25% aqueous MeCN at 20 mL/min) in order to remove the unwanted diastereomer. The pure fractions were concentrated to give the product as a clear oil. Yield **14a**: 0.67 g (55 %); ES-MS m/z 493.4 $[M+H]^+$; UV λ_{max} 215, 256 nm.

Preparation of Compound 15a

10 Compound **14a** was treated according to deprotection procedure D to provide compound **15a**.

Preparation of Compound 18a

Compounds **15a** and **16** were coupled and characterized as described in Pettit *et. al. J. Chem. Soc. Perk I*, 1996, 859.

15 Preparation of [(2S)-2-[(1R,2R)-3-[(1R,2S)-2-hydroxy-1-methyl-2-phenylethyl] amino]-1-methoxy-2-methyl-3-oxopropyl]-pyrrolidine-1-carboxylic acid-*tert*-butyl ester (**25**)

t-Boc-dolaproine **20** and (1S,2R)-norephedrine **21** were combined in the presence of DEPC and triethylamine according to General procedure A (see also U.S. Patent 5,635,483 to Pettit et al.) to provide compound **25**.

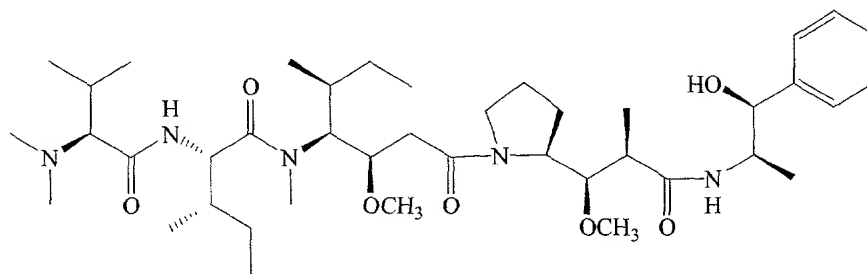
20 Preparation of *N,N*-dimethyl-L-valyl-*N*-[(1S,2R)-4-[(2S)-2-[(1R,2R)-3-[(1R,2S)-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1S)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, (**29a**, Auristatin E).

Compound **18a** and **25** were combined in the presence of trifluoroacetic acid 25 in methylene chloride (1:1), followed by treatment with DEPC and triethylamine, to

provide compound **29a** using procedure C. The synthesis of this compound is also reported in U.S. Patent No. 5,635,483, and Pettit et al., *Anti-Cancer Drug Des.* 1998, 243.

EXAMPLE 2

PREPARATION OF COMPOUND **29b**



Preparation of Compound **14b**

According to coupling procedure B, this peptide was prepared using compounds **12** and **13b**. After concentration of the reaction mixture, the residue was purified by C₁₈ preparative-HPLC (Varian Dynamax column 21.4 mm x 25 cm, 5 μ , 100 Å, using an isocratic run of 75% aqueous MeCN at 20 mL/min) in order to remove the unwanted diastereomer. The pure fractions were concentrated to a clear oil. Yield **14b**: 0.67 g (55 %); R_f 0.32 (4:1 hexanes-acetone); UV λ_{max} 215, 256 nm; ¹H NMR (CDCl₃) δ 7.14-7.40 (5 H, m), 6.19 (1 H, d, J = 9.3 Hz), 5.09 (2 H, s), 4.53 (1 H, dd, J = 6.6, 9.3 Hz), 3.34 (3 H, s), 2.97 (3 H, s), 2.25-2.50 (2 H, m), 1.50-1.78 (3 H, m), 1.46 (9 H, s), 0.99 (3 H, d, J = 6.9 Hz), 0.96 (3 H, d, J = 6.9 Hz), 0.89 (3 H, t, J = 6.9 Hz), 0.83 (3 H, t, J = 7.2 Hz).

Preparation of Compound **15b**

Compound **14b** was treated according to deprotection procedure D to provide compound **15b**.

Preparation of Compound 18b

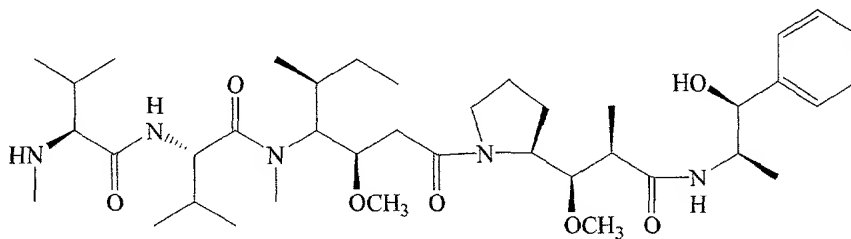
Compounds **15b** and **16** were combined using procedure A to provide compound **18b**: Yield **18b**: 0.19 g (82 %); ES-MS m/z 500.5 $[M+H]^+$; R_f 0.12 (4:1 hexanes-acetone); UV λ_{max} 215 nm.

5 Preparation of *N,N*-dimethyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[[*(1R,2S*)-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidiny]-2-methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-isoleucinamide, (**29b**)

Following general procedure C, compounds **18b** and **25** were combined in
10 the presence of trifluoroacetic acid in methylene chloride, followed by treatment with DEPC and triethylamine. The reaction mixture was purified by preparative-HPLC (C₁₈-RP Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN in 100 mM aqueous triethylammonium carbonate from 10 to 100 % in 40 min followed by 20 min at 0 %
15 buffer, at flow rate 20 mL/min). The product was isolated as an off-white solid after concentration of the desired HPLC fractions. Yield **29b**: 85 mg (60 %); ES-MS m/z 746.5 $[M+H]^+$; UV λ_{max} 215 nm.

EXAMPLE 3

PREPARATION OF COMPOUND 31a



Preparation of Compound 19a

Compounds **15a** and **17** were combined using procedure A to provide compound **19a**: 107 mg (50 %); ES-MS m/z 694.7 $[M+H]^+$; R_f 0.64 (1:1 hexanes-EtOAc); UV λ_{max} 215, 265 nm.

5 Preparation of Compound 30a

Following general procedure C, compounds **19a** and **25** (prepared as described in Example 1) were combined in the presence of trifluoroacetic acid in methylene chloride, followed by treatment with DEPC and triethylamine. The reaction mixture was monitored by HPLC and after 4h water was added and the layers separated.

- 10 After drying ($MgSO_4$), the contents were filtered and solvent removed to give compound **30a** that was used in the next step without further purification. Yield **30a**: 127 mg (91%); ES-MS m/z 940.9 $[M+H]^+$.

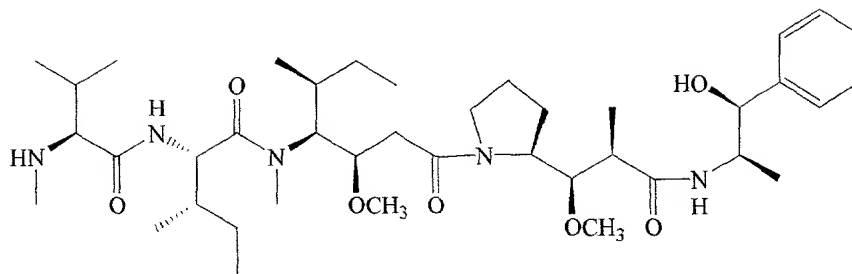
Preparation of *N*-methyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[(1*R*,2*S*)-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-

- 15 methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, (**31a**)

- 20 The Fmoc-protected peptide was treated with diethylamine according to deprotection procedure E. A complete reaction was observed by HPLC after 12 h. The reaction mixture was concentrated to an oil and purified by preparative-HPLC (C_{18} -RP Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN in water 25 to 70% in 5 min followed by 30 min at 70%, at a flow rate of 20 mL/min). The desired fractions were pooled and concentrated to give an off-white solid. Yield **31a**: 37 mg (38 %); ES-MS m/z 718.7 $[M+H]^+$; UV λ_{max} 215 nm.

EXAMPLE 4

PREPARATION OF COMPOUND 31b



Preparation of Compound 19b

Compounds **15b** and **17** were combined using procedure A to provide compound **19b**. Yield **19b**: 0.50 g (73 %); UV λ_{max} 215, 265 nm.

Preparation of Compound 30b

Following general procedure C, compounds **19b** and **25** (prepared as described in Example 1) were combined in the presence of trifluoroacetic acid in methylene chloride, followed by treatment with DEPC and triethylamine. The reaction mixture was purified by preparative-HPLC (C₁₈-RP Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN in water 25 to 70% in 5 min followed by 30 min at 70%, at a flow rate of 20 mL/min). The product was isolated as an off-white solid after concentration of the desired HPLC fractions. Yield **30b**: 64 mg (53 %); ES-MS m/z 955.2 [M+H]⁺; UV λ_{max} 215, 265 nm.

Preparation of *N*-methyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[(1*R*,2*S*)-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, (**31b**)

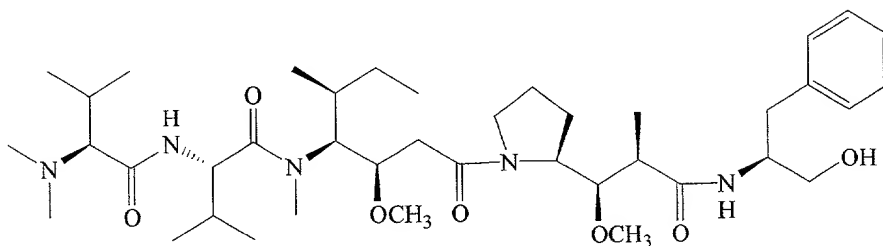
The Fmoc-protected peptide **30b** was treated with diethylamine according to deprotection procedure E. A complete reaction was observed by HPLC after 2 h. The reaction mixture was concentrated to an oil. Excess ether was added resulting in a white

precipitate and the contents were cooled to 0 °C for 3 h. The product was filtered and the solid dried under high vacuum. Yield **31b**: 47 mg (95 %); ES-MS m/z 732.8 $[M+H]^+$; UV λ_{\max} 215 nm.

5

EXAMPLE 5

PREPARATION OF COMPOUND **32**



Preparation of [(2*S*)-2-[(1*R*,2*R*)-3-[[1*S*]-hydroxymethyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-pyrrolidine-1-carboxylic acid- *tert*-butyl ester (**26**)

10 The dipeptide **26** was synthesized from *t*-Boc-dolaproine **20** and (*S*)-(-)-2-amino-3-phenyl-1-propanol (L-phenylalaninol) **22** according to General procedure A. Yield **26**: 0.50 g (55 %); ES-MS m/z 421.0 $[M+H]^+$; R_f 0.24 (100% EtOAc); UV λ_{\max} 215, 256 nm. ^1H NMR (CDCl_3) δ 7.14-7.40 (5 H, m), 6.19 (1 H, d, J = 7.8 Hz), 4.11-4.28 (1 H, m), 3.44 (3 H, s), 3.20-3.84 (5 H, m), 2.80-3.05 (2 H, m), 2.20-2.38 (2 H, m), 1.65-1.98 (4

15 H, m), 1.48 (9 H, s), 1.16 (3 H, d, J = 5.7 Hz).

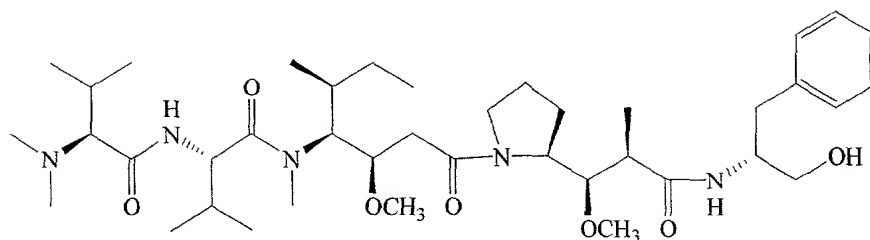
Preparation of Compound **32**

Dipeptide **26** and tripeptide **18a** are combined in the presence of trifluoroacetic acid in methylene chloride, following a coupling reaction with DEPC and triethylamine as described in general procedure C. The reaction mixture is purified under

20 usual preparative-HPLC measures as previously described for other peptide compounds.

EXAMPLE 6

PREPARATION OF COMPOUND 33



[(2*S*)-2-[(1*R*,2*R*)-3-[(1*R*)-hydroxymethyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-

5 oxopropyl]-pyrrolidine-1-carboxylic acid- *tert*-butyl ester (27)

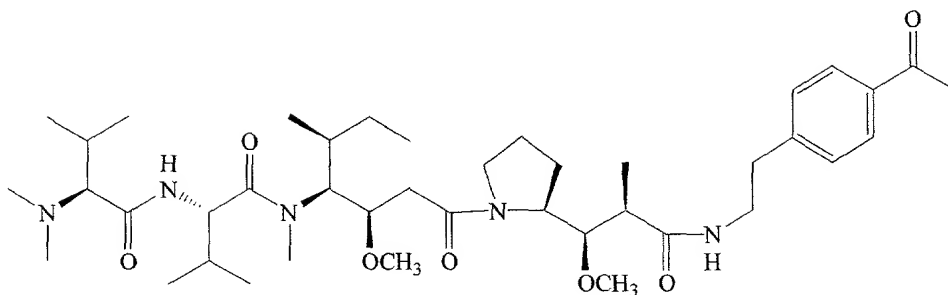
The dipeptide **27** was synthesized from *t*-Boc-dolaproine **20** and (*R*)-(+)-2-amino-3-phenyl-1-propanol (D-phenylalaninol) **23** according to General procedure A. Yield **27**: 0.53 g (58 %); ES-MS m/z 421.0 $[M+H]^+$; R_f 0.24 (100% EtOAc); UV λ_{max} 215, 256 nm. 1H NMR ($CDCl_3$) δ 7.14-7.40 (5 H, m), 6.19 (0.5 H, d, $J = 5.4$ Hz), 5.75 (0.5 H, d, $J = 5.4$ Hz), 4.10-4.23 (1 H, m), 3.38 (3 H, s), 3.10-3.85 (4 H, m), 2.82-2.96 (2 H, m), 2.10-2.36 (2 H, m), 1.66-1.87 (4 H, m), 1.47 (9 H, bs), 1.16 (1.5 H, d, $J = 6.6$ Hz), 1.08 (1.5 H, d, $J = 5.7$ Hz).

Compound 33

Dipeptide **27** and tripeptide **18a** are combined in the presence of 15 trifluoroacetic acid in methylene chloride, followed by a coupling reaction with DEPC and triethylamine as described in general procedure C. The reaction mixture is purified under usual preparative-HPLC measures as previously described for other peptide compounds.

EXAMPLE 7

PREPARATION OF COMPOUND 34



Preparation of Compound 28

- 5 Dipeptide **28** is prepared by reacting Boc-dolaproine **20** and *p*-acetylphenethylamine (US patent 3445518, 1969) according to coupling procedure B. After concentration of the reaction mixture, the residue is purified by reverse phase preparative-HPLC or via SiO₂ chromatography.

Preparation of *N,N*-dimethyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[(4-

- 10 acetylphenyl)ethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, (**34**)

- Dipeptide **28** and tripeptide **18a** are combined in the presence of trifluoroacetic acid in methylene chloride, following a coupling reaction with DEPC and triethylamine as described in general procedure C. The reaction mixture is purified under
15 usual preparative-HPLC measures as previously described for other peptide compounds.

EXAMPLE 8

PREPARATION OF COMPOUND 36

Preparation of *N,N*-dimethyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[(1*R*)-2-oxo-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide (35)

Pyridinium chlorochromate (PCC) (13.6 mg, 0.06 mmol, 4.5 eq.) was added to a solution of Auristatin E (**29a**) (10 mg, 0.014 mmol, 1 eq.) in CH₂Cl₂ (2 mL) and pyridine (50 μL). The reaction mixture was stirred at room temperature for 3 h. HPLC analysis of the reaction mixture showed complete conversion of the 10.2 min peak into a new 11.3 min peak with different UV spectrum (max 245 nm, shoulder 280 nm). The product was purified by flash chromatography on a silica gel column (120 × 12 mm) in a step gradient of MeOH in CH₂Cl₂ from 0 to 10 %. After concentration in vacuum, the residue was triturated with hexane to give white solid. Yield **35**: 6.4 mg (64 %); *R_f* 0.3 (CH₂Cl₂/MeOH, 10/1); UV λ_{max} 215, 245 nm. HRMS *m/z*: found 730.5119 [M+H]⁺. C₄₀H₆₈N₅O₇ requires 730.5108.

Preparation of Hydrazone 36

Compound **36** was prepared from compound **35** by reaction with 30 eq. of maleimidocaproylhydrazide for 3 days according to General Procedure F. Yield **36**: 2.4 mg (43 %) of colorless glass; ES-MS *m/z* 937.1 [M+H]⁺; UV λ_{max} 215, 240 (shoulder) nm.

EXAMPLE 9

PREPARATION OF COMPOUND 38

Preparation of levulinic ester of auristatin E (37)

Levulinic acid (2.5 μL, 0.025 mmol, 5 eq.) was added to a solution of auristatin E (**29a**, 3.6 mg, 0.005 mmol, 1 eq.) in anhydrous CH₂Cl₂ (1 mL), followed by DCC (5 mg, 0.025 mmol, 5 eq.) and DMAP (1 mg, cat). After reacting for overnight at

room temperature, analysis by C₁₈ RP-HPLC revealed formation of a new, more hydrophobic product. Precipitated DCU was filtered off. The resulting keto ester was isolated using preparative chromatography on silica gel in a step gradient of MeOH in CH₂Cl₂ from 0 to 10%. Yield **37**: 3.3 mg (80.5 %) of colorless glass; *R_f* 0.35 (CH₂Cl₂/MeOH, 10/1); ES-MS *m/z* 830 [M+H]⁺; UV λ_{max} 215 nm.

Preparation of Hydrazone **38**

Compound **38** was prepared from compound **37** and maleimidocaproylhydrazide (2 eq.) according to General Procedure F, reaction time – 6 h. Yield **38**: 1.3 mg (46 %) of white solid, ES-MS *m/z* 1037 [M+H]⁺. UV λ_{max} 215 nm.

EXAMPLE 10

PREPARATION OF COMPOUND **40a**

Preparation of 4-acetyl-benzoic ester of auristatin E (**39a**)

4-Acetylbenzoic acid (23.5 mg, 0.14 mmol, 2 eq.) was added to a solution of auristatin E (**29a**, 50 mg, 0.07 mmol, 1 eq.) in anhydrous CH₂Cl₂ (5 ml), followed by DCC (30 mg, 0.14 mmol, 2 eq.) and DMAP (5 mg, cat). After reacting overnight at room temperature, analysis by C₁₈ RP-HPLC revealed formation of a new, more hydrophobic product. Precipitated DCU was filtered off. The resulting keto ester was isolated using preparative chromatography on silica gel in a step gradient of MeOH in CH₂Cl₂ from 0 to 10%. The product was eluted with 5% MeOH in CH₂Cl₂. Yield **39a**: 57 mg (90 %) of white solid; *R_f* 0.43 (CH₂Cl₂/MeOH, 10/1); UV λ_{max} 215, 250 nm. HRMS *m/z*: found 878.5669 [M+H]⁺. C₄₉H₇₆N₅O₉ requires 878.5643. Anal. calcd for C₄₉H₇₅N₅O₉ × H₂O: C, 65.67; H, 8.66; N, 7.81. Found: C, 66.05; H, 8.61; N, 7.80.

Preparation of Hydrazone **40a**

Compound **40a** was prepared from compound **39a** and maleimidocaproylhydrazide (3 eq.) according to General Procedure F, reaction time – 12 h.

Yield **40a**: 3 mg (65 %) of solid; UV λ_{max} 215, 295 nm. HRMS m/z : found 1085.6665 $[M+H]^+$. $C_{59}H_{89}N_8O_{11}$ requires 1085.6651.

EXAMPLE 11

PREPARATION OF COMPOUND **40b**

Preparation of 4-acetyl-benzoic ester of *N,N*-dimethyl-L-valyl-*N*-[(1*S*,2*R*)-4-[(2*S*)-2-[(1*R*,2*R*)-3-[[*(1R,2S)*-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-isoleucinamide, (**39b**)

Compound **39b** was prepared from compound **29b** by reaction with 4-acetobenzoic acid in the presence of DCC, DMAP in CH_2Cl_2 as described in the Example 10. Yield **39b**: 10.7 mg (80 %) of white solid; R_f 0.37 ($CH_2Cl_2/MeOH$, 10/1); UV λ_{max} 215, 250 nm. HRMS m/z : found 892.5775 $[M+H]^+$. $C_{50}H_{78}N_5O_9$ requires 892.5800.

Preparation of Hydrazone (**40b**)

Compound **40b** was prepared from compound **39b** and maleimidocaproylhydrazide (5 eq.) according to General Procedure F, reaction time – 18 h. Yield **40b**: 4.8 mg (50 %) of solid; ES-MS m/z : 1099 $[M+H]^+$; UV λ_{max} 215, 295 nm.

EXAMPLE 12

PREPARATION OF COMPOUND **42**

Preparation of Compound **42**

To a solution of **39a** (5 mg, 0.0056 mmol) in DMSO (100 μ L) anhydrous MeOH (0.9 mL) was added, followed by 1 % AcOH/MeOH (10 μ L). 3-Bromoacetamido)propionyl hydrazide (**41**) (0.025 mmol, 4.5 eq.) was added to the solution. The reaction was left at room temperature for 24 h. C_{18} -RP HPLC analysis revealed the formation of a new compound with a lower retention time. DMSO (1 mL)

was added to the reaction mixture and methanol was removed under reduced pressure. The residue was directly loaded onto a C₁₈-RP column for preparative HPLC purification (Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN in 100 mM TEAA buffer, pH 7.0, from 10 to 95 % at flow rate 4 mL/min). The appropriate fractions are concentrated under reduced pressure, co-evaporated with acetonitrile (4 \times 25 mL), and finally dried in deep vacuum. Yield **42**: 4.0 mg (66 %) of solid; ES-MS *m/z*: 1084 [M+H]⁺, 1086; UV λ_{max} 215, 295 nm.

EXAMPLE 13

10 CYTOTOXICITY STUDY: COMPOUNDS **29a** AND **39a**

In vitro cytotoxicity experiments on several cell lines were performed to compare the activities of auristatin E (**29a**) with the ketoester **39a**. The ester proved to be cytotoxic on a wide panel of cell lines, ranging from as cytotoxic as auristatin E (**29a**) on L2981 cells, to being approximately 17-times less cytotoxic on Daudi Burkitt's lymphoma cells (Table 1).

Table 1

CELL LINES USED FOR THE EVALUATION OF MAB-AURISTATIN E CONJUGATES, AND THE RELATIVE CYTOTOXIC EFFECTS OF AURISTATIN E (**29a**) AND THE KETOESTER DERIVATIVE **39a**

Cell Line	Tumor Type	Antigen Expression (MFI)			IC ₅₀ (nM)	
		LeY	CD40	CD30	29a	39a
L2987	lung	620	171	7	1.0	0.9
Kato III	gastric	94	neg.	neg.	0.7	5
SKBR3	breast	2985	20	88	1.5	6
AU565	breast	869	19	57	1.5	6
Daudi	Burkitt's lymphoma	37	72	neg.	0.9	15
L428	Hodgkin's lymphoma	74	16	76	1.5	13
L540	Hodgkin's lymphoma	99	0	223	1.5	16
IM-9	Multiple myeloma	132	47	40	3.5	50
Karpas	ALCL	59	1	246	1.2	13

To obtain the results shown in Table 1, the cells were exposed to drugs for 1-2 hours, and the cytotoxic effects were determined 72 hours later by the incorporation of ³H-thymidine into DNA. Antigen expression was determined by flow cytometry and is expressed in terms of mean fluorescence intensity (MFI).

5 Further studies were undertaken to explore the stability of **39a** in human serum, and it was shown that there was no detectable hydrolysis after 120 hours incubation at 37°C. Furthermore, prolonged exposure of **39a** to purified esterases from human, rhesus monkey, guinea pig and rabbit livers failed to demonstrate any conversion of **39a** to auristatin E (**29a**). Thus it appears that **39a** may not be an auristatin E prodrug, but instead
10 exhibits activity as a benzylic ester.

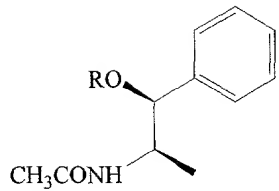
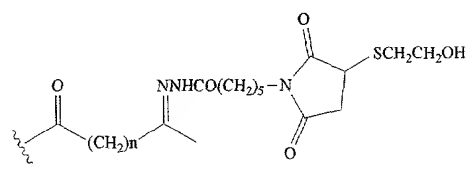
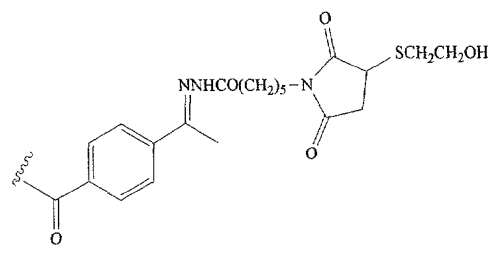
EXAMPLE 14

STRUCTURAL EFFECTS ON HYDRAZONE HYDROLYSIS

15 In order to analyze the structural requirements for efficient hydrazone hydrolysis under mildly acidic conditions, a series of norephedrine derivatives (Table 2) related in structure to the C-terminus of a pentapeptide of the invention were prepared.

Table 2

COMPOUNDS TESTED FOR HYDRAZONE HYDROLYSIS KINETICS

Compound	R =
	
43 (n = 2)	
44 (n = 3)	
45 (n = 5)	
46	

- 5 Condensation of N-acetylnorephedrine with various ketoacids in the presence of DCC led to the formation of corresponding ketoesters. Upon reaction with maleimidocaproylhydrazide, hydrazones were formed. HPLC was used for stability determination at pH 5 and 7.2 at 37°C. 2-Mercaptoethanol (2 eq.) was added to quench the reactive maleimide functionalities forming compounds **43-46** *in situ*. The mercaptoethanol adducts **43-45** were unstable at both pH 5 and 7.2 (Table 3). In contrast, the benzylic hydrazone **46** hydrolyzed quite slowly at pH 7.2, but underwent hydrolysis at pH 5 with a half-life of 5 hours. The product formed upon hydrolysis of **46** was the parent ketoester.
- 10

Table 3

HYDROLYSIS OF THIOETHER-MODIFIED HYDRAZONES OF NOREPHEDRIN AT 37 °C.

Hydrazone	t _{1/2} at pH 5.0	t _{1/2} at pH 7.2
43	< 2 minutes	8 hours
44	< 2 minutes	2 hours
45	< 2 minutes	< 10 minutes
46	5 hours	60 hours

5

EXAMPLE 15

PREPARATION OF mAb-DRUG CONJUGATES

A solution of monoclonal antibody (mAb) (5-10 mg/mL) in phosphate buffered saline, pH 7.2, is reduced with dithiothreitol (65 eq.) at 37 °C for 45 minutes.

- 10 Separation of low molecular weight agents is achieved by size exclusion chromatography on a Sephadex G25 column, and the sulfhydryl content in the mAb is determined using 5,5'-dithiobis(2-nitrobenzoic acid) as described previously (Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) "Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)-a reexamination" *Anal. Biochem.* 94, 75-81). There are typically between 6-9 free sulhydryl
- 15 groups per mAb.

- To the reduced mAb is added acetonitrile (12% final vol) followed by drug- (reactive linker) **36**, **38**, **40a**, or **40b** in a 1 mM solution of 9:1 acetonitrile:DMSO so that the final drug concentration is 1.9-fold higher than that of the mAb sulfhydryl groups. After 60 min at room temperature, glutathione (1 mM final concentration) and oxidized
- 20 glutathione (5 mM final concentration) are added, and the solution is allowed to stand for an additional 10 min. Unbound drug is removed by repeated ultrafiltration (Amicon, YM30 filter) until there is no evidence of low molecular weight agents in the filtrate. The concentrated protein is gel filtered on Sephadex G25, a small portion of polystyrene beads

is added to the pooled protein fraction, and the solution is sterile filtered using 0.4 micron filters. The protein concentration is determined at 280 nm ($E_{0.1\%}^{1.4}$).

Free drug concentration is determined by reversed-phase chromatography against standard solutions of drug-(reactive linker) standards (C_{18} Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN in 5 mM ammonium phosphate buffer, pH 7.0, from 10 to 90 % in 10 min followed by 90 % MeCN for 5 min at flow rate 1 mL/min). Typically, there is less than 0.5 % free drug in the purified conjugate preparations. Bound drug is quantified by hydrolyzing the linked drugs from the mAb with 1 volume of pH 1.5 aqueous HCl buffer for 15 minutes at room temperature, neutralizing the solution with borate buffer at pH 8, and analyzing drug concentration using reversed phase HPLC as indicated above. There are typically between 4-7 drugs/mAb.

EXAMPLE 16

HYDROLYSIS OF THIOETHER-MODIFIED LINKERS AND IMMUNOCONJUGATES

15 Synthesis of Thioethers of compounds 36, 38, 40a, and 40b.

Mercaptoethanol adducts of compounds 36, 38, 40a, and 40b were prepared *in situ* by a reaction of the corresponding maleimido hydrazones (0.3 mM in 10 % aqueous PBS, 10 % DMSO) with 2 eq. of mercaptoethanol for 15 min at room temperature. HPLC analysis (C_{18} Varian Dynamax column, 5 μ , 100 Å, linear gradient of MeCN from 10 to 90 % in 5 mM ammonium phosphate buffer, pH 7.0, in 10 min, then 90 % of MeCN for 5 min at flow rate 1 mL/min) showed new peaks with retention times of 0.3 – 1 min less than for parent hydrazones. Reaction mixtures were directly used for hydrolysis kinetics.

Kinetics of the hydrazone bond hydrolysis for thioethers of compounds 36, 38, 40a, and 40b.

25 Thioethers solutions (0.3 mM in 10 % aqueous PBS, 10 % DMSO) were adjusted to a final salt concentration of 30 mM with either 100 mM NaOAc, pH 5.0, or 100 mM Na_2HPO_4 , pH 7.2, then incubated at 37°C in a temperature controlled autosampler of

the HPLC system. Aliquots (10 μ L) were taken at timed intervals and the disappearance of starting material was monitored by HPLC at 215 nm (C_{18} Varian Dynamax column, 5 μ , 100 Å, linear gradient from 10 to 90 % of MeCN in 5 mM ammonium phosphate buffer, pH 7.0, in 10 min, then 90 % of MeCN for 5 min at flow rate 1 mL/min). The results were expressed as percents of starting hydrazones. Half-lives of hydrazones were calculated from these curves and are listed in Table 4.

Immunoconjugate Hydrolysis Kinetics

Conjugates (1 mg/mL) in 10 % aqueous DMSO, 50 mM buffer (either NaOAc, pH 5.0, or 50 mM Na_2HPO_4 , pH 7.2) were incubated at 37 °C in a temperature controlled autosampler of the HPLC system. Aliquots (100 μ L) were taken at timed intervals and the formation of a drug (keto-ester) was monitored by HPLC at 250 nm (C_{18} Varian Dynamax column, 5 μ , 100 Å, linear gradient from 10 to 90 % of MeCN in 5 mM ammonium phosphate buffer, pH 7.0, in 10 min, then 90 % of MeCN for 5 min at flow rate 1 mL/min). Free drug was quantitated by integrating the corresponding peak (positively identified by comparison to a standard). The results were presented as percents of total drug released. Half-lives of immunoconjugates were calculated from these curves and are listed in Table 4.

Table 4

HYDROLYSIS OF THIOETHER-MODIFIED LINKERS AND IMMUNOCONJUGATES AT 37 °C.

Conjugate	$t_{1/2}$ at pH 5.0	$t_{1/2}$ at pH 7.2
BR96-doxorubicin	3.5 hours	>120 hours
BR96-S-36	50 hours	>500 hours
Thioether of 38	4 hours	9 hours
Thioether of 40a	8 hours	110 hours
BR96-S-40a	15 hours	250 hours

EXAMPLE 17

PREPARATION OF hBR96-S-**42** CONJUGATE.

To 450 μ L of PBS, pH 7.2, containing 3.0 mg hBR96 was added 50 μ L of
5 100 mM dithiothreitol (DTT) in water. The mixture was incubated at 37 $^{\circ}$ C for 30 min. Excess DTT was removed from the reduction reaction by elution through a PD10 column (Pharmacia) with PBS containing 1 mM DTPA. The number of free thiols per antibody was determined to be 8.5, by measuring the protein concentration (A_{280} , assuming the absorbance of a 1 mg/mL solution = 1.4) and the A_{412} of an aliquot of the protein treated
10 with DTNB, assuming a molar extinction coefficient of 14,150.

The pH of the 1.2 mL reduced antibody solution was raised to 8.7 by the addition of 150 μ L 500 mM Na-Borate/500 mM NaCl, pH 8.7. A solution containing a 12-fold excess of compound **42** over antibody was prepared in 150 μ L DMSO. The solution of compound **42** was added to the reduced antibody solution with vigorous stirring and the
15 reaction mixture incubated at room temperature for about 3 hours. The reaction mixture was quenched by the addition of sufficient 200 mM sodium tetrathionate to make the solution 1 mM.

The quenched reaction mixture was purified by immobilization on an ion exchange matrix, rinsing with a partial organic solvent solution, elution from the matrix
20 and buffer exchange into PBS. Thus, the quenched reaction mixture was diluted to 35 mL in 25 mM Tris, pH 9 ("Eq. Buffer"), then loaded onto an EMD TMAE column equilibrated in Eq. Buffer. The immobilized conjugate was rinsed with a mixture of 20% acetonitrile/80% Eq buffer, then eluted with 0.5 M NaCl/19 mM Tris, pH 9. Fractions were analyzed by size exclusion chromatography and pooled. The eluted conjugate was
25 concentrated by centrifugal ultrafiltration, then eluted through a PD 10 column in PBS, producing 1.2 mL of a 2.0 mg/mL solution (based on A_{280}). Elution of conjugate through a C_{18} column indicated that any unconjugated drug was below the detection level (~ 0.2 μ M). Treatment of the conjugate with pH 1.7 buffer for 15 min, followed by C_{18} HPLC analysis indicated that the drug was covalently conjugated to the antibody. Comparison of the

HPLC peak area from the hydrolyzed conjugate with an standard curve of **39a** determined the level of the drug attached to a given quantity of conjugate; comparison with the antibody concentration determined by A_{280} , gave a ratio of 3.2 drug/hBR96. Treatment of H3396 cells with this conjugate inhibited cell growth with an IC_{50} of about 1.5 $\mu\text{g/mL}$.

5

EXAMPLE 18

IN VITRO CYTOTOXICITY DATA FOR mAb-S-36

The cytotoxic effects of the mAb-S-36 conjugates on L2987 human lung
10 adenocarcinoma cells (strongly BR96 antigen positive, weakly S2C6 antigen positive) and
on Kato III human gastric carcinoma cells, (strongly BR96 antigen positive, S2C6 antigen
negative) are shown in Figures 12A, 12B and 12C. These figures show the *in vitro*
cytotoxicity of drugs and mAb-drug conjugates on (A) and (B) L2987 human lung
adenoma cells, and (A) and (C) Kato III human gastric carcinoma cells. L2987 cells are
15 positive for the antigens recognized by BR96 (LeY) and S2C6 (CD40), but express the
LeY antigen at higher levels. Kato III cells are positive for the LeY antigen and negative
for CD40. Cells were exposed to the conjugates for 2 hours, washed, and the cytotoxic
effects were determined 3 days later using a thymidine incorporation assay.

Auristatin E (**29a**) and the ketone derivative **35** were at least 100-fold more
20 cytotoxic than doxorubicin on L2987 and Kato III cells (Figure 12A). On both cell lines,
the BR96-S-36 conjugate displayed increased potency compared to S2C6-S-36, suggesting
some degree of immunological specificity. The low potency of BR96-S-36 is most likely
due to its stability at pH 5 (Table 4), suggesting that only a small portion of the conjugated
drug is released under the assay conditions.

25

EXAMPLE 19

IN VITRO CYTOTOXICITY DATA FOR mAb-S-40a

The cytotoxic effects of the conjugates on several cell lines are shown in
5 Figures 13A, 13B, 13C and 13D. These figures show the cytotoxic effects of auristatin E
and auristatin E-containing conjugates on L2987 human lung adenocarcinoma cells (Figure
13A); SKBR3 (Figure 13B); AU565 human breast carcinoma cells (Figure 13C), and
various hematologic cell lines treated with AC10-S-40a (Figure 13D). In all assays, cells
10 were exposed to the drugs for 2 hours, washed, and the cytotoxic activities were measured
72 hours using either (A and D) ^3H -thymidine incorporation or (B and C) XTT dye
reduction.

On L2987 cells (Figure 13A), BR96-S-40a was significantly more cytotoxic
than S2C6-S-40a, reflecting increased LeY antigen expression compared to CD40 (Table
1). The effects of the AC10-S-40a conjugate on several hematologic cell lines are shown in
15 Figure 13D. The most sensitive cells were L540 Hodgkin's lymphoma and Karpas
anaplastic large cell lymphoma, both of which strongly express the CD30 antigen. The
L428 cell line, which is intermediate in CD30 antigen expression, was affected to a lesser
extent by the AC10-S-40a conjugate. The effects appeared to be immunologically specific,
since the antigen-negative control cell line, Daudi, was quite insensitive to the conjugate.
20 One of the interesting findings in this study was that IM-9, a strong expresser of the CD30
antigen (Table 1), was also insensitive to the AC10-S-40a conjugate. Upon further
analysis, it was found that unlike the other cell lines, IM-9 did not internalize bound
conjugate. Thus, these results demonstrate that activity requires not only specific binding,
but also antigen internalization.

EXAMPLE 20

IN VIVO ACTIVITIES OF mAb-S-40a CONJUGATES.

Nude mice with subcutaneous L2987 human lung adenocarcinoma or 3396 human breast carcinoma xenografts were injected with conjugates or drug according to the schedule shown in Figures 14A and 14B. The BR96-S-40a conjugates bound to the tumor lines, while the IgG-S-40a did not. All animals were monitored daily for general health, and every 3-8 days for weight and tumor growth. Tumor volumes were estimated using the formula: (longest dimension) x dimension perpendicular²/2. There was no toxicity associated with the mAb-S-40a conjugates. The effects were compared to those of auristatin E at the maximum tolerated dose. Estradiol implants were used to sustain the growth of 3396 tumors. The implants released estradiol over a period of 60 days, at which point the experiment was terminated.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually incorporated by reference. For example, the book in *Comprehensive Organic Transformations, A Guide to Functional Group Preparations*, Second Edition, Richard C. Larock, John Wiley and Sons, Inc., 1999, and particularly the references cited therein, is incorporated herein by reference for all purposes.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.